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Prophylactic and therapeutic intervention of Punta Toro virus (*Phlebovirus*, Bunyaviridae) infection in hamsters with interferon alfacon-1

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Abstract

Punta Toro virus (PTV) is a member of the Bunyaviridae family, genus *Phlebovirus*, related to the highly pathogenic Rift Valley fever virus (RVFV). It produces a disease in hamsters that models severe Rift Valley fever (RVF) in humans. The recent outbreak of RVF in Kenya stresses the need to identify prophylactic and therapeutic measures for preventing and treating severe forms of disease. To this end, interferon (IFN) alfacon-1 (consensus IFN-α) was evaluated in cell culture against RVFV and PTV, and in the hamster PTV infection model. Survival outcome following treatment initiated pre- and post-virus challenge and the suppression of viral burden and liver disease in infected hamsters was determined. Pre-treatment of cell cultures with IFN alfacon-1 induced marked antiviral activity against both viruses. Intraperitoneal treatment of hamsters initiated 4 h prior to infection with PTV was highly protective and greatly limited liver disease and systemic and liver viral burden. Complete protection from a highly lethal challenge dose was afforded by treatment initiated 36 h following viral inoculation. Although efficacy was much reduced, IFN alfacon-1 therapy was still beneficial when started as late as 3–5 days post-virus exposure. These studies suggest that IFN alfacon-1 may be an effective treatment for early intervention following infection with RVFV.

Keywords: Punta Toro virus; Phlebovirus; Interferon; Interferon alfacon-1; Antiviral; Hamster

1. Introduction

Several viruses of the *Phlebovirus* genus, Bunyaviridae family, are known to cause significant disease in humans. These viruses are maintained in nature in wild or domesticated mammals and are transmitted through feeding of phlebotomous insects. Consequently, phleboviral disease is closely linked to factors that influence host and vector populations. Infection by certain strains of sandfly fever virus (SFFV) has historically been a disease of considerable importance to the military. During World War II, many of the ~19,000 soldiers infected with SFFV required medical attention and hospitalization (Hertig and Sabin, 1964). Although rarely fatal, SFFV infection can result in acute intense fever, severe myalgias, nausea, vomiting, abdominal pain and diarrhea (Sabin et al., 1944). Toscana is another phlebovirus of medical importance that is one of the leading causes of meningitis in Italy and other neighboring European countries (Charrel et al., 2005; Echevarria et al., 2003; Peyrefitte et al., 2005). Punta Toro virus (PTV), a New World phlebovirus, causes an acute febrile illness that is generally self-limiting (Anderson et al., 1990). The most significant phleboviral pathogen is Rift Valley fever virus (RVFV). Epizootic outbreaks in domesticated ungulates can have devastating economic effects (Gerdes, 2002). In humans, RVFV infection can be quite severe in the form of viral hemorrhagic fever and encephalitis (Alrajhi et al., 2004; Peters and Meegan, 1981). Recent outbreaks in sub-Saharan Africa, Madagascar, Yemen, Egypt and Saudi Arabia have resulted in considerable morbidity and mortality (Centers for Disease Control and Prevention, 1994, 2000a,b, 2007; Morvan et al., 1991). Reflecting the concern of public health officials, RVFV has been classified as a "Category A" pathogen by the National Institute of Allergy and Infectious

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Diseases (NIAID, 2002), and has received dual "Select Agent" status by the Department of Health and Human Services and the United States Department of Agriculture.

Interferon (IFN) alfacon-1, trade name Infergen®, is a bio-optimized recombinant IFN-α that contains the most frequently occurring amino acids present among the non-allelic subtypes, of which there are at least 13 (Roberts et al., 1998). Compared to the naturally occurring IFN-α proteins, IFN alfacon-1 has demonstrated enhanced in vitro antiviral activity (Blatt et al., 1996). It is most commonly used for the treatment of hepatitis C virus infections in combination with the broad-spectrum antiviral, ribavirin (Sjogren et al., 2005; Tong et al., 1997; Witthoeft et al., 2007). Although IFN alfacon-1 activity is lacking in mice, we and others have previously demonstrated varying degrees of efficacy produced by treatment of acute viral diseases in different hamster infection models (Fish et al., 1985, 1986; Gowen et al., 2005; Julander et al., 2007; Morrey et al., 2004a,b). Collectively, these studies have indicated that the recombinant IFN alfacon-1 designed through consensus sequences present in human IFN-α proteins is reactive in the hamster system. While IFN alfacon-1 activity is likely to be considerably more potent in humans, activity in hamsters facilitates pre-clinical development for the treatment of human viral infections modeled in hamsters.

Although it is likely that all phleboviruses encode a type I IFN antagonist to subdue antiviral defenses in their maintenance hosts, thus allowing for sufficient replication and successful transmission, effective treatment of RVFV infection in rhesus monkeys with human INF-α has been previously reported (Morrill et al., 1989). In that study, Morrill and colleagues treated monkeys 24 h prior to or 6 h after infection. Since the early presence of type I IFN is associated with lack of clinical illness and survival in the rhesus model (Morrill et al., 1990), it is likely that efficacy would be reduced with delayed administration of IFN therapy. Further evidence supporting the need for rapid induction of type I IFN for the effective control of acute phleboviral infection and disease was recently shown in the hamster PTV infection model (Perrone et al., 2007). The PTV Adames strain is highly lethal in hamsters, while the Balliet strain of the virus produces a mild self-limiting infection (Anderson et al., 1990). The nonstructural protein encoded by the S segment of the Adames strain was found to disrupt type I IFN induction to a greater degree than that of the Balliet strain, likely contributing to the observed Adames strain lethality and its initial suppression of serum type I IFN levels following infection (Perrone et al., 2007). Thus, the treatment of patients having advanced RVFV infections with IFN-α may not provide much benefit. On the other hand, the encouraging data in rhesus monkeys would support the use of IFN-α in the event of accidental laboratory infection or possibly as a prophylactic measure for high-risk individuals during a severe epidemic.

IFN alfacon-1 has not been previously evaluated for the treatment of phleboviral infections. Here we investigated the prophylactic and therapeutic antiviral activity of IFN alfacon-1 against PTV infection in hamsters, as a model for severe RVFV pathogenesis and disease in humans. The ability of IFN alfacon-1 to protect animals from death and mitigate viral burden and liver disease was examined. Moreover, the effects of handling and stress associated with treatment of infection were also investigated.

2. Materials and methods

2.1. Cells and animals

The monkey kidney cell lines Vero 76 and LLC-MK_2 were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in minimal essential medium (MEM) supplemented with 0.18% $NaHCO_3$ and 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Female 7–8-week-old golden Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for 2–3 days prior to use. Animal procedures complied with USDA guidelines and were approved by the Utah State University Institutional Animal Care and Use Committee.

2.2. Viruses

PTV, Adames strain, was obtained from Dr. Dominique Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The virus used for cell-based studies was from a stock prepared following four passages of the original virus through LLC-MK_2 cells. This virus was inoculated into hamsters for the production of a high titer preparation derived from pooled liver homogenates, and this stock was used for the in vivo challenge studies. The RVFV vaccine strain, MP-12, was provided by Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX). The RVFV stock was prepared from virus passaged twice in Vero 76 cells.

2.3. Test materials

IFN alfacon-1 (trade name Infergen®), lot number A014896, was provided by Intermune, Inc. (Brisbane, CA) and had a reported activity of 1×10^9 units/mg. The material was at a concentration of 30 μg/ml and was further diluted to the appropriate concentrations with sterile saline. Ribavirin was supplied by ICN Pharmaceuticals Inc. (Costa Mesa, CA). Both drugs were prepared in MEM for cell culture experiments and in sterile saline solution for in vivo delivery.

2.4. Cell-based antiviral testing

Predetermined amounts of virus that would visually yield 80–95% CPE by day 6 in preliminary virus titration experiments were prepared in culture medium containing 2% FBS. Vero 76 cells plated in 96-well microtiter plates were exposed to one-half $\log_{10}$ dilutions of IFN alfacon-1 for 10–15 h prior to the addition of virus. Ribavirin was added to test wells at the time of infection. For toxicity determinations, drugs were incubated with cells in the absence of viral challenge. Plates were incubated at 37 °C, 5% CO_2, until virus-infected control wells presented with >80% cytopathic effect (CPE) by visual analysis, at which time plates were scored visually for CPE and toxic-

ity. Following visual analysis, the plates were incubated with 0.034% neutral red (NR) dye solution for 2 h at 37 °C, 5% CO_2. Afterwards, the NR was removed and the cells were washed twice with PBS and air-dried prior to extraction of the vital dye with absolute ethanol buffered with Sorenson's citrate buffer. Samples were read at 540 nm on a BioTek EL 800 microplate reader (BioTek, Winooski, VT) and the absorbance values were expressed as percent of untreated, uninfected controls. The 50% effective concentration (EC_{50}) and the 50% cytotoxic concentration (CC_{50}) values were determined by regression analysis and selectivity index (SI) values were calculated as the CC_{50}/EC_{50}. Virus yield reduction assays were performed to verify NR assay results. The concentration of drug required to reduce virus yield by 10-fold (EC_{90}) was determined by regression analysis.

2.5. In vivo PTV challenge studies

Groups of 15 hamsters (25–30 for the placebo group) were treated by intraperitoneal (i.p.) injection with varying doses of IFN alfacon-1, ribavirin, or sterile saline placebo, as indicated. IFN alfacon-1 treatments were initiated 4 h prior to or at various times after subcutaneous (s.c.) inoculation with 50 plaque-forming units (PFU) of PTV. Treatments were administered once a day for 6–7 days, and ribavirin, included as a positive control, was given twice daily for 6–7 days, starting 4 h pre-inoculation. Livers and sera were harvested from five animals from each group (up to 10 for the placebo group) sacrificed on day 4 of infection. Livers were scored on a scale of 0–4 for hepatic icterus; 0 being normal and 4 being maximal yellow coloration. Serum was assayed for alanine aminotransferase (ALT) activity. Infectious viral loads were determined for both liver and serum samples. The remaining animals in each group were observed for 21 days. For comparison, three sham-infected hamsters were included as normal controls in order to establish baselines for the tested parameters. Toxicity evaluations with similar tested doses and schedules of IFN alfacon-1 and ribavirin were not warranted since they have previously been shown to be well tolerated (Gowen et al., 2005).

In the therapeutic efficacy studies, 10–20 hamsters were challenged as indicated above and drug and placebo treatments were initiated 36–120 h post-infection with PTV. Survival over a 21-day period was assessed. For the time course study, groups of three hamsters per day (four for the day-4 group) were challenged with 50 PFU of PTV. One group of animals was sacrificed each day for 7 days. Livers, spleens, and serum were collected from each animal. Serum was collected for assaying ALT activity and virus titers were determined for liver and spleen homogenates and serum samples as described below.

2.6. Liver, spleen and serum virus titers

Virus titers were assayed using a previously described infectious cell culture assay (Gowen et al., 2006b). In brief, liver or spleen homogenates or serum samples were serially diluted and added to triplicate wells of LLC-MK_2 cells plated in 96-well microplates. Viral CPE was determined 6–7 days post-virus exposure and the 50% endpoints were calculated as described (Reed and Muench, 1938). The assay detection range was 2.75–9.5 $\log_{10}$ cell culture 50% infectious doses ($CCID_{50}$)/g of tissue or 1.75–8.5 $\log_{10}$ $CCID_{50}$/ml of serum. In samples with undetectable virus levels, a value of 2.75 or 1.75 $\log_{10}$, respectively, was assigned for statistical analysis. Conversely, in cases where virus exceeded the detection range, a value of 9.5 or 8.5 $\log_{10}$ was assigned.

2.7. Measurement of serum ALT

Assessment of ALT activity present in serum samples is an indirect method for determining liver disease associated with hepatotropic PTV infection. Serum ALT activity levels were measured using the ALT (SGPT) Reagent Set from Pointe Scientific Inc. (Lincoln Park, MI), as recommended by the Manufacturer. Reagent volumes were adjusted for analysis in 96-well microplates.

2.8. Statistical analysis

The log-rank test was used for comprehensive survival analysis using JMP statistical software (SAS, Cary, NC). The Mann–Whitney *U*-test (two-tailed) was performed to analyse differences in mean day of death, virus titers, serum ALT levels, and hepatic icterus.

3. Results

3.1. Cell culture activity of IFN alfacon-1 against PTV and RVFV

RVFV has previously demonstrated sensitivity to the effects of type I IFN in cell culture (Peters et al., 1989). Here we examined the antiviral activity of IFN alfacon-1 against both RVFV and PTV. As seen in Table 1, pretreatment with IFN alfacon-1 resulted in EC_{50} values in the picogram (pg) range as determined by the NR CPE reduction assay. Visible reduction of CPE in IFN alfacon-1-treated cells was also evident (data not shown). Further, high levels of activity were verified by virus yield reduction with mean EC_{90} values of 32 and <3.5 pg/ml against PTV and RVFV, respectively (Table 1). Ribavirin was included as a positive control in these studies to ascertain the treatability of the infections. Notably, the CC_{50} values for ribavirin against PTV were lower than those seen for RVFV. This discrepancy was likely due to the longer incubation period required for maximal CPE development following PTV infection (5–6 days) compared to RVFV (3 days). Moreover, RVFV testing was performed in near-confluent monolayers, whereas PTV studies were done in less confluent cells in order to promote greater CPE.

3.2. Prophylactic IFN alfacon-1 treatment of PTV infection in hamsters

PTV infection in hamsters has been well characterized and is believed to recapitulate many aspects of disease seen in human cases of severe disease caused by the highly pathogenic RVFV

Table 1
Antiviral activity of IFN alfacon-1 against PTV and RVFV in cell-based assays[a]

Drug[b]	Virus	Neutral red uptake assay			Virus yield reduction		
		$CC_{50} \pm$ S.D.	$EC_{50} \pm$ S.D.	SI[c]	$CC_{50} \pm$ SD	$EC_{90} \pm$ S.D.	SI[c]
IFN alfacon-1 (pg/ml)	PTV	>3200	12 ± 1.7	>267	>3200	32 ± 10	>99
	RVFV	>3200	<2.1 ± 2.0	>1500	>3200	<3.5 ± 4.3	>914
Ribavirin (μg/ml)	PTV	397 ± 285	28 ± 17	14	>803 ± 341	43 ± 4.7	19
	RVFV	>510 ± 433	11 ± 6.8	>48	>1000	6.5 ± 3.0	>155

[a] Data represent the means and standard deviations from three independent experiments in Vero 76 cells.
[b] Cells were exposed to IFN alfacon-1 for 10–15 h prior to the addition of virus; ribavirin was introduced at the time of infection.
[c] SI, calculated as mean CC_{50}/EC_{50}, is the selectivity (therapeutic) index.

(Anderson et al., 1990; Fisher et al., 2003). Utilizing this model system of acute phleboviral disease, we investigated the ability of IFN alfacon-1 to induce an antiviral state in hamsters that would serve to protect them from a lethal infectious inoculum of PTV. In the initial trial, we examined treatment doses of 20 and 5 μg/kg/day, previously found to be optimal and suboptimal, respectively, against Pichinde virus infection in hamsters (Gowen et al., 2006c). As shown in Fig. 1A, both treatments, initiated 4 h prior to virus inoculation, afforded complete protection from an infectious dose that killed 100% of hamsters by day 9 of the infection in the placebo group. Ribavirin, included to confirm the treatability of the PTV inoculum, was also protective. All drug treatments effectively reduced systemic and liver virus burdens measured on day 4 of the infection, with the 20-μg/kg/day dose of IFN alfacon-1 having the most dramatic effect (Fig. 1B and C). Liver disease parameters were also reduced by treatment. Baseline ALT levels were observed for all IFN alfacon-1 and ribavirin treatment groups, whereas 3 of the 10 placebo-treated hamsters had elevated values (Fig. 1D). In addition, liver discoloration was significantly reduced in all treatment groups compared to the animals that received the saline placebo (Fig. 1E). Taken together, the data indicate that both the 20 and 5 μg/kg/day doses of IFN alfacon-1 were highly effective at preventing death and limiting viral burden and liver disease.

A second experiment was conducted to determine the lower limits of protection resulting from IFN alfacon-1 treatments. Doses of 5, 1, 0.5 and 0.1 μg/kg/day were tested using the same administration schedule employed in the initial trial. Remarkably, as little as 0.5 μg/kg/day of IFN alfacon-1 offered complete protection from an inoculum of PTV that was 95% lethal in placebo-treated animals (Fig. 2A). Although there was some hint of protection seen at the lowest dose (0.1 μg/kg/day), the difference was not statistically significant compared to animals receiving the placebo. As in the first experiment, the 5-μg/kg/day dose was highly effective at abrogating viral replication and minimizing liver disease (Fig. 2B–E). The 1-μg/kg/day treatment was found to be equally effective with antiviral activity and liver-protective effects waning at the 0.5 and 0.1 μg/kg/day doses. Thus, although the 0.5 μg/kg/day dose was effective in the context of protecting hamsters from death, the ability of this treatment to control viral burden and prevent liver damage was reduced. Interestingly, two hamsters that received ribavirin failed to survive the PTV challenge; however, the animals that perished survived appreciably longer than those treated with placebo (Fig. 2A). This delayed disease progression is reflected by the lack of serum and liver virus, and barely detectable liver disease on day 4 of the infection (Fig. 2B–E).

3.3. Therapeutic intervention of PTV infection in hamsters with IFN alfacon-1

From a practical standpoint, only severe phleboviral infections are likely to require antiviral therapy. This type of intervention would be started sometime after the onset of illness and manifestations of severe disease. To this end, examining the therapeutic efficacy of IFN alfacon-1 is of great importance from the perspective of clinical treatment. As expected, the efficacy of IFN alfacon-1 diminished as the time of treatment initiation was delayed (Fig. 3A). Treatment with the highly efficacious prophylactic dose of 5 μg/kg/day, started 36 h post-infection, resulted in 100% survival. When treatments began 48, 60 or 72 h after challenge, significant protection compared to the placebo-treated hamsters (5% survival) was still evident as 70, 60 and 60% survival was observed, respectively (Fig. 3A). Ribavirin, dosed as indicated in the first two studies, offered full protection (data not shown). In this experiment, saline placebo treatments were initiated at 120 h post-viral challenge. Despite 95% lethality in this treatment group, the hamsters that succumbed to the infection persisted longer than those from the placebo groups in the first two experiments (treatment initiated 4 h prior to PTV inoculation), as shown by superimposition of the survival curves from all three of the challenge studies (Fig. 3B). By log-rank analysis, survival differed significantly depending on time at which treatment was initiated. Compared to the first two experiments, the delayed placebo therapy resulted in highly variable times of death spanning a period of 13 days. Collectively, the data suggest that significant protection against PTV infection can be elicited by delayed IFN alfacon-1 therapy and that handling stress associated with treatment may influence disease progression.

3.4. Temporal analysis of viral burden and liver disease during the course of PTV infection

To attempt to correlate efficacy data with disease markers in the hamster PTV infection model, we set out to define the times at which viral replication and liver disease became apparent dur-

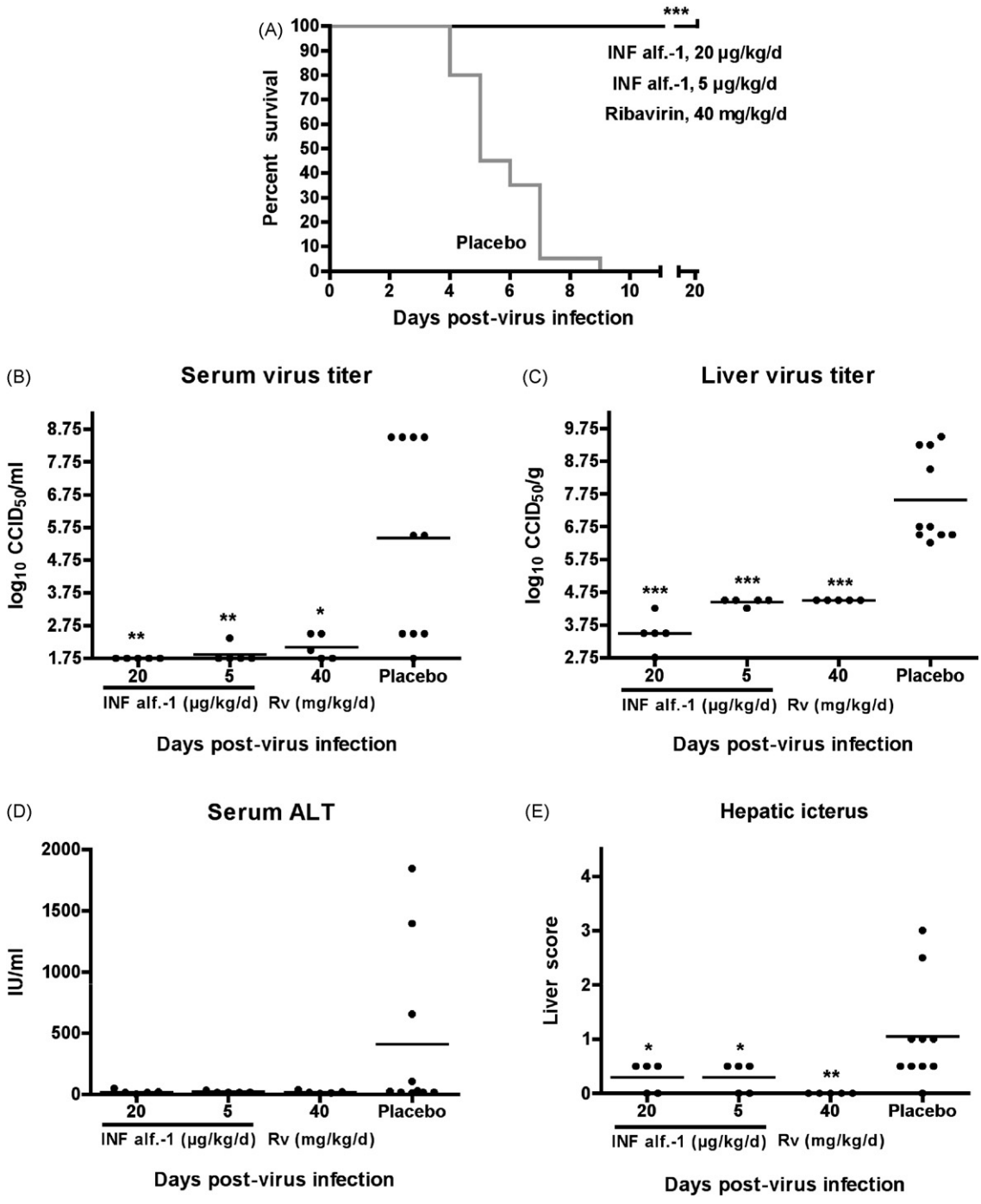

Fig. 1. Prophylaxis of lethal PTV infection and disease in hamsters with IFN alfacon-1 and ribavirin. Groups of 15 hamsters (30 for the placebo) were treated with 20 or 5 μg/kg/day of IFN alfacon-1, 40 mg/kg/day of ribavirin, or saline placebo. The IFN and placebo treatments were once daily, for 7 days, and ribavirin was given twice daily, for 7 days. All treatments were initiated 4 h prior to virus inoculation. Ten hamsters per group (20 for the placebo) were observed daily and (A) survival was plotted over a period of 21 days. The remaining 5 hamsters in each group (10 for the placebo) were sacrificed on day 4 of the infection for analysis of infectious (B) serum and (C) liver virus titers, (D) ALT levels and (E) hepatic icterus (increasing in severity from 0 to 4). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to placebo-treated animals. IFN alfacon-1, IFN alf.-1; ribavirin, Rv.

ing the course of infection. Hamsters were challenged with an infectious dose of PTV that normally produces >80% lethality in adult hamsters. Three or four animals were sacrificed daily and virus titers and serum ALT were determined for each. The earliest that virus could be detected was on day 2 with >5 $\log_{10}$ of virus detected in the serum and liver tissue in one of three hamsters (Fig. 4A and B). This, and one other animal, also had measurable spleen virus (Fig. 3C). On day 3 of the infection, one hamster had greater than 7 $\log_{10}$ of serum, liver and spleen virus (Fig. 4A–C). Both of the other hamsters were devoid of systemic

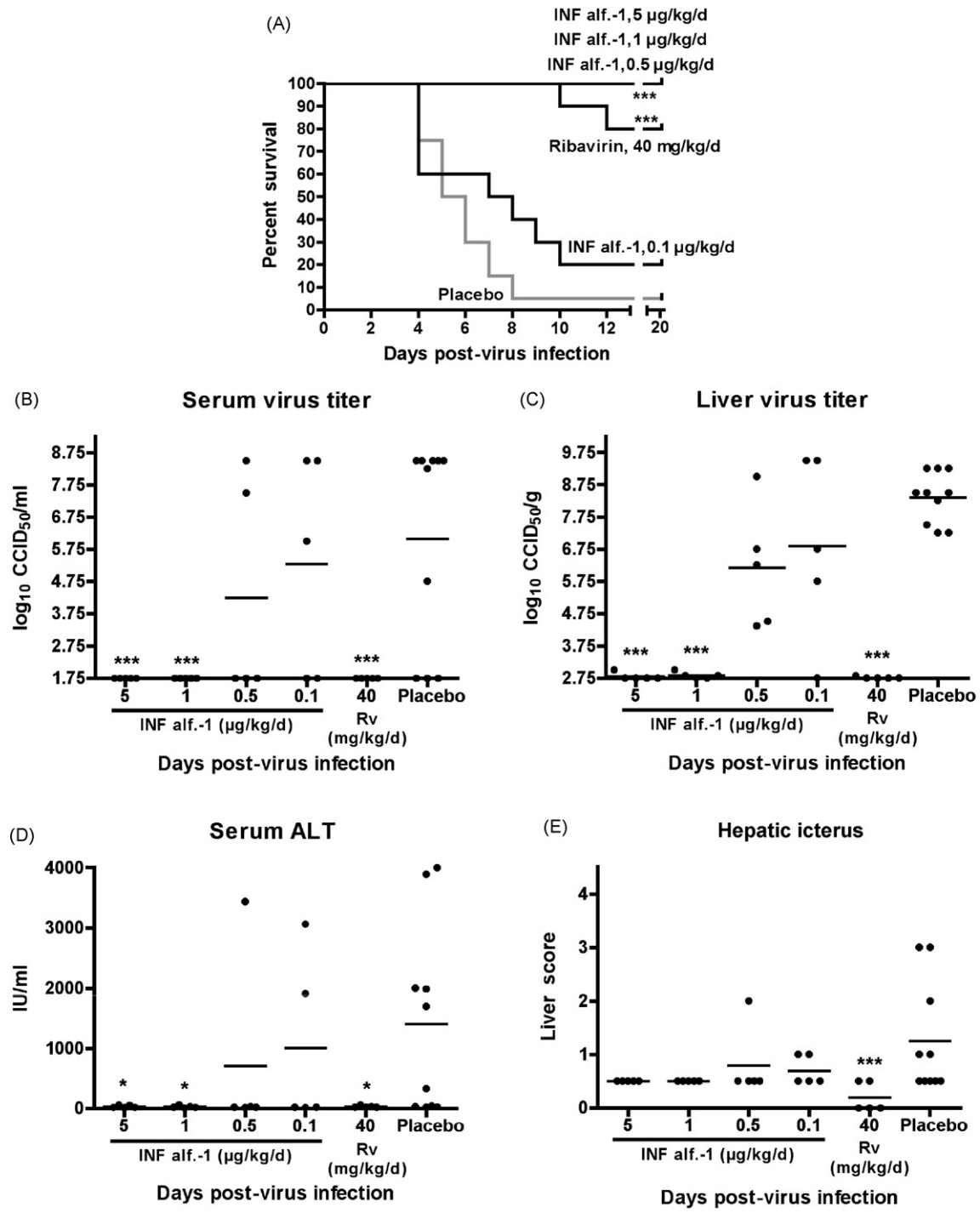

Fig. 2. Protection limits of IFN alfacon-1 in preventing PTV mortality and disease. Groups of 15 hamsters (25 for the placebo) were treated with 5, 1, 0.5, or 0.1 µg/kg/day of IFN alfacon-1, 40 mg/kg/day of ribavirin, or saline placebo. The IFN and placebo treatments were once daily, for 6 days, and ribavirin was given twice daily, for 6 days. All treatments were initiated 4 h prior to virus inoculation. Ten hamsters per group (20 for the placebo) were observed daily and (A) survival was plotted over a period of 21 days. The remaining five hamsters per group were sacrificed on day 4 of the infection for analysis of infectious (B) serum and (C) liver virus titers, (D) ALT levels and (E) hepatic icterus (increasing in severity from 0 to 4). $*P < 0.05$; $***P < 0.001$ compared to placebo-treated animals. IFN alfacon-1, IFN alf.-1; ribavirin, Rv.

virus, but had low levels of spleen virus; one also had detectable levels of liver virus. By day 4, three of the four animals in the group had >9.5 $\log_{10}$ of systemic virus, with the single animal that had considerably less serum virus also having the lowest levels of liver and spleen viral titers (Fig. 4A–C). The rest of the animals had large viral loads in liver and spleen tissue. On day 5 of the infection, one of the three animals died prior to the time of sacrifice. The two that survived had peak serum, liver and spleen virus titers close to or >9 $\log_{10}$. A single animal survived in the day-6 sacrifice group and this animal was free

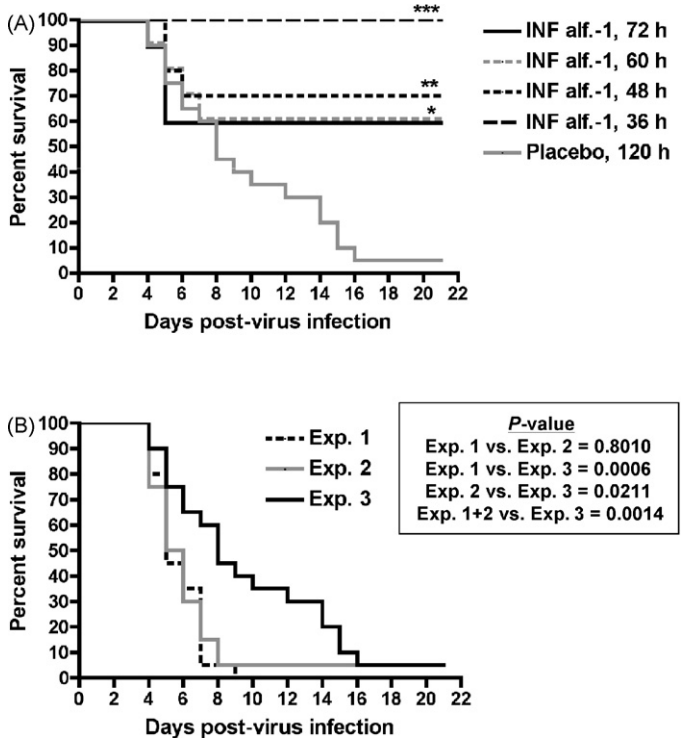

Fig. 3. Therapeutic intervention of advanced PTV infection in hamsters with IFN alfacon-1. Groups of 10 hamsters (20 for the placebo) were treated with 5 μg/kg/day of IFN alfacon-1, 40 mg/kg/day of ribavirin, or saline placebo. IFN alfacon-1 therapy was initiated 36, 48, 60 or 72 h post-infectious challenge, and administered once daily, for 6 days. The placebo treatment began 120 h after the infection. Hamsters were observed daily and (A) survival was plotted over a period of 21 days. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to placebo-treated animals. (B) Handling stress associated with treatment influences disease outcome of PTV infection in hamsters. In the first two experiments, placebo treatment began 4 h prior to infection with PTV. In the third experiment, therapy was not initiated until 120 h after challenge. The survival data from the three challenge studies were compared by log-rank analysis and the resulting *P*-values are shown. IFN alfacon-1, IFN alf.-1; experiment, exp.

of detectable serum and liver virus (Fig. 4A and B). This animal, however, had a modest spleen viral burden. As in the day-6 group, only a single hamster survived to day 7. This animal was also clear of detectable serum virus but had considerable spleen viral burden, with moderate liver virus. The data suggest that virus replication began to ramp up as early as the second day of infection in some animals.

In the analysis of ALT as indicator of liver disease, significant rises were not evident until day 4, at which the three animals with high levels of virus in all sampled tissues had greater than 1000 IU/ml (Fig. 4D). The single animal that presented with only low levels of virus had a baseline level of ALT. Both animals in the day-5 group had ALT levels indicative of severe liver disease. Interestingly, the animals that survived to days 6 and 7 of the infection had normal levels of ALT. This was likely a consequence of the more controlled viral replication seen in these animals, which had no systemic viral burden and only limited, if any, liver virus (Fig. 4A and B). These data indicate that elevation of ALT levels as a marker for liver dysfunction due to PTV infection does not present until day 4 and correlates with high serum viral loads.

3.5. IFN alfacon-1 therapeutic limit of protection and impact of treatment initiation on survival outcome

Since we could not define the point in time at which therapeutic intervention was no longer protective against PTV infection in the previous experiment (Fig. 3A), an additional study where treatment was delayed until 5 days post-challenge was conducted. Considering the results from the first three experiments where there was evidence of more rapid disease progression in hamsters that started receiving placebo early during the course of infection (Fig. 3B), saline treatments were initiated on day 2 or 5 to investigate whether the observed handling effect could also be resolved under such conditions. As demonstrated in Fig. 5, comparison of the survival curves for the days 2 and 5 placebo groups showed a significant improvement in outcome for those animals where treatment was delayed. For those animals where initiation of treatment with 5 μg/kg/day of IFN alfacon-1 was delayed out to 4 or 5 days post-infection, 60% survived compared to 20% for the day-5 placebo group (Fig. 5). For the group that started receiving drug on day 3 of infection, 70% protection was afforded. By log-rank analysis, the day-5 placebo group comparison with the day-3 IFN alfacon-1 group, previously shown to be significant (Fig. 3A), was just above the threshold with a *P*-value of 0.0519. For the day-2 placebo group, where uniform lethality with a steep survival curve was seen, all comparisons to the drug-treated groups resulted in statistically significant differences. This group, however, is not the most appropriate for comparison since there appears to be a clear effect related to the time of treatment initiation.

4. Discussion

The recent RVF outbreak in Kenya places emphasis on the importance of identifying prophylactic and therapeutic measures for the prevention and treatment of cases of severe disease. Many of the cases reported to the Kenya Ministry of Health presented with severe illness and bleeding, resulting in 118 deaths and a high case-fatality rate of 29% (CDC, 2007). Various drug candidates have shown efficacy in the treatment of RVFV infections in pre-clinical animal models (Canonico et al., 1984; Morrill et al., 1989, 1991; Peters et al., 1986), with only ribavirin having progressed to clinical evaluation (P. Rollin, presented at the Treatment of Viral Hemorrhagic Fever workshop, Bethesda, MD, 25–27 February 2007). Notably, studies with IFN-α (Morrill et al., 1989) and IFN-γ (Morrill et al., 1991) have focused on prophylaxis of RVFV with initiation of treatment beginning within a day of the infection. In our study, preventive treatment and therapeutic intervention of phleboviral infection in hamsters with IFN alfacon-1 was evaluated.

Recent studies investigating the therapeutic potential of IFN alfacon-1 in hamster viral infection models have produced varying degrees of success. When initiated 4 h prior to challenge with Pichinde arenavirus (PICV), 10 μg/kg/day (qd, 7 days) was found to be optimal, protecting 80% of hamsters from a lethal infection, while a dose of 1 μg/kg/day was suboptimal, protecting only 40% of the animals (Gowen et al., 2005). In the current study, employing a similar treatment schedule, as little as

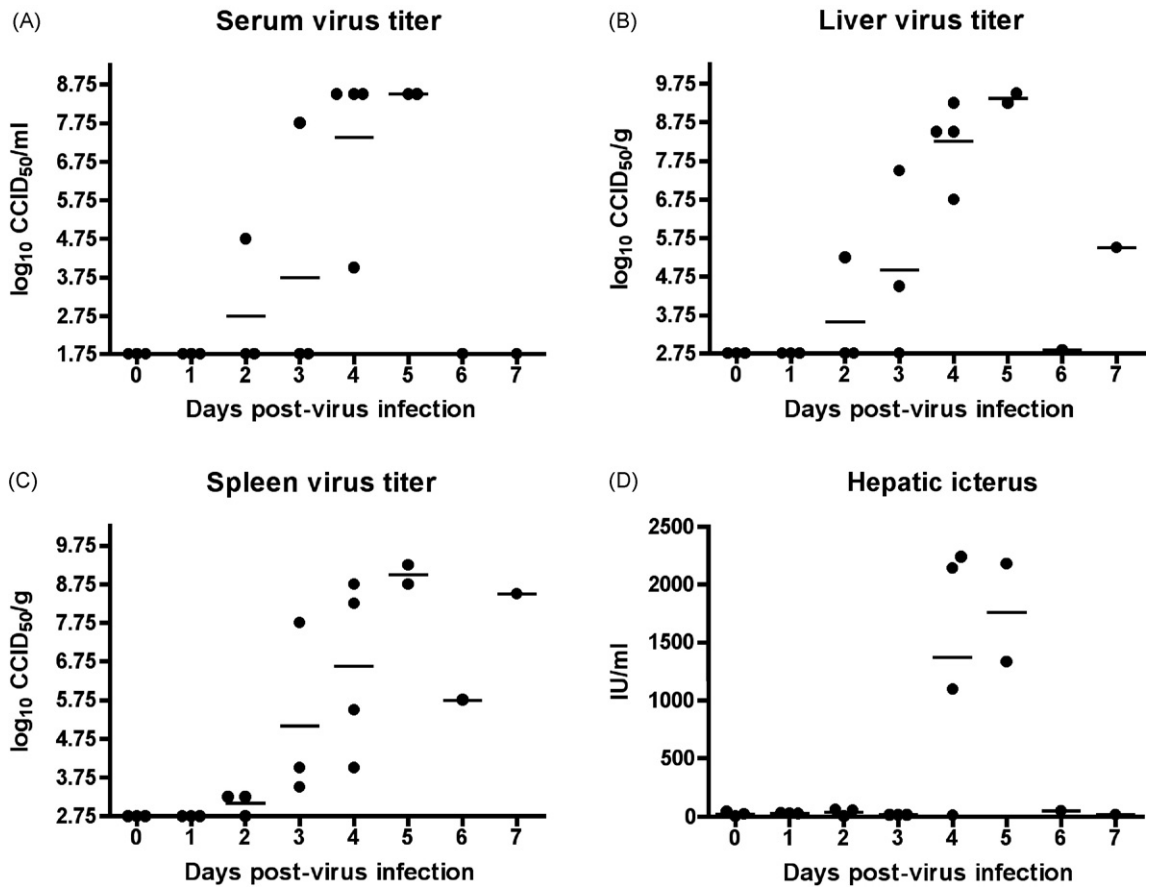

Fig. 4. Liver, spleen and serum virus burden and ALT levels during the course of PTV infection in hamsters. Tissue and serum samples were collected on the indicated days post-virus challenge for groups of three hamsters, with the exception of the day-4 group, which had 4 animals. Due to death prior to time of sacrifice, samples could only be obtained for 2, 1 and 1 hamsters for the day 5, 6 and 7 groups, respectively. (A) Serum, (B) liver and (C) spleen virus titers, and (D) ALT values were determined as described in Section 2. Data points represent viral load or ALT level for a single hamster with the group mean represented by a black bar.

0.5 μg/kg/day of IFN alfacon-1 protected 100% of PTV-infected hamsters from an infectious inoculum that killed 95% of animals receiving saline placebo. It is also important to note that >80% protection could not be achieved in the PICV model with doses up to 40 μg/kg/day (B. Gowen, unpublished data). The reduced antiviral affect observed against PICV infection in hamsters, which serves as a model for Lassa fever, is consistent with the reported resistance of Lassa virus to IFN, whereas RVF disease, modeled by the PTV infection, is much more sensitive to the effects of IFN (Peters et al., 1989).

Detailed characterization of animal systems is essential when trying to correlate data obtained in animal models to the human condition. If one is to attempt to make inferences to the treatment of human disease, it is important to determine the time at which disease markers become apparent during the course of infection. Here we examined in a temporal fashion, virological and clinical disease parameters that serve as indicators of disease progression in the hamster PTV infection model. Measurable viral titers were seen as early as day 2 with increasing loads through day 5. Since certain animals will succumb to PTV infection well into the second week, it is difficult to determine whether the two survivors in the days 6 and 7 groups are in the process of becoming ill or, less likely, recovering from the infection. We found that delaying the initiation of IFN alfacon-1 therapy to 3 days post-infection resulted in a barely significant level of protection. Although there was an apparent therapeutic effect in groups of hamsters receiving IFN alfacon-1 starting as late as 4–5 days after infection, the number of animals used was insufficient to achieve statistical significance. Regardless of these encouraging results, the therapeutic application of IFN-based drugs for treating advanced stages of RVFV infections may be limited if early induction of type I IFN is a defining event in the outcome of phleboviral infection (Morrill et al., 1990; Perrone et al., 2007).

The extended survival times and shallower survival curves observed for PTV-infected hamsters receiving placebo beginning on day 5, as compared to those that were treated starting 4 h prior to, or 2 days after challenge, suggests that stress associated with the restraint and i.p. injections early on contributes significantly to the disease process. In the second therapeutic study, the day-2 placebo initiation of treatment time was selected for comparison with day 5 since it coincided with the earliest time at which there was evidence of viral burden. Presumably, handling stress associated with treatment starting at this point in time would have a considerable impact on accelerating disease progression. Consistent with the data presented here, increased mortality due to stress associated with the handling of the mice during the course of infection has been reported in the mouse PTV infection model (Gowen et al., 2006a).

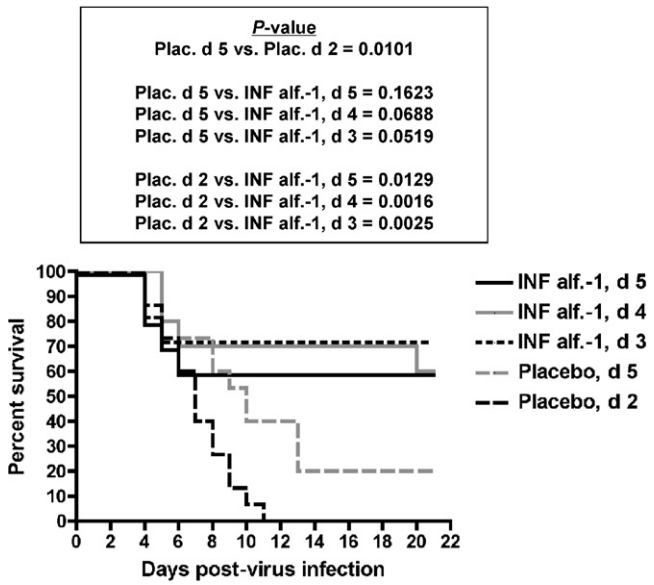

Fig. 5. Therapeutic limits of IFN alfacon-1 and influence of treatment initiation time on disease outcome. Groups of 10 hamsters (15 for the placebo) were treated with 5 μg/kg/day of IFN alfacon-1 or saline placebo. IFN alfacon-1 therapy was initiated 3, 4 or 5 days post-infectious challenge, and administered once daily, for 6 days. The placebo treatment began either 2 or 5 days after infection. Hamsters were observed daily and survival was plotted over a period of 21 days. The survival data were compared by log-rank analysis and the resulting *P*-values are shown. IFN alfacon-1, INF alf.-1; placebo, plac.

It is difficult to compare the effects of the various human IFN-based drugs in hamsters since differing degrees of cross-reactivity may account for differences in potency and inaccurately reflect the human response. In the case of acute diseases such as RVF, treatment of patients seeking medical attention due to manifestations of severe disease are likely to have advanced infections that may be difficult to treat with IFN alone. It may be beneficial to combine IFN treatment with ribavirin for patients presenting with signs of hemorrhagic fever. However, it is questionable as to the effectiveness of such an approach since the addition of IFN alfacon-1 to ribavirin treatment of hamster PICV infection did not improve outcome when administered 48 h or later during the infection (Gowen et al., 2006c). Experiments in the rhesus monkey model evaluating how late after RVFV challenge INF-α therapy can be initiated and still offer protection are needed to better address this question.

There is strong evidence for the use of IFN-α in the event of accidental laboratory infections where therapeutic intervention can be made within a short period of time. In nonhuman primates challenged with RVFV and treated with IFN-α 24 h prior to, or 6 h after infection, no signs of clinical disease were evident and systemic viral burden was non-existent or dramatically reduced (Morrill et al., 1989). Our data also support early intervention, as complete protection was afforded when IFN alfacon-1 treatment was initiated within 36 h of infection. It may be possible during a severe outbreak to prevent infection in high-risk individuals such as those living near swamps or working as herdsmen, or anyone involved in the slaughtering or processing livestock. Studies investigating the duration of the antiviral state in animal models of acute phleboviral infection following IFN alfacon-1 treatment would be needed to determine the most appropriate frequency of dosing. Unless the cost associated with the administration of IFN-α therapies can be greatly reduced, particularly for developing countries, such an approach would not likely be feasible.

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