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Characterization of murine Caraparu *Bunyavirus* liver infection and immunomodulator-mediated antiviral protection

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Summary

A rapid, peripheral disease model utilizing the *Bunyavirus*, Caraparu, was established in mice for the evaluation of antiviral therapy with immunomodulators. 4–6-week-old B6C3F1 female mice, inoculated intraperitoneally with virus, developed coagulative liver necrosis and died between 4–6 days after infection. This Caraparu disease model was relatively resistant to treatment with immunomodulators, such as ABMP, Ampligen, α-interferon (IFN-α) or β-interferon (IFN-β). However, a significant increase in median survival time (MST) was consistently observed upon treatment with γ-interferon (IFN-γ). The nucleoside analog – ribavirin – was highly effective against Caraparu virus in repeated treatment schedules begun on either day −1, day 0, or day +1 of infection. Ribavirin gave little protection when initiation of treatment was delayed until day +2. However, combined treatment with IFN-γ, starting on day 0 and ribavirin starting on day +2, significantly reduced mortality.

Murine Caraparu *Bunyavirus*; Immunomodulator; Ribavirin

Introduction

The *Bunyaviruses* are enveloped – RNA viruses which are approx. 80 to 100 nm in diameter (Fenner et al., 1974). Their genome consists of three segments

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of single-stranded RNA of negative polarity (Bishop, 1985). More than 220 *Bunyaviruses* have been identified (Shope, 1985). Some of the *Bunyaviruses* cause significant human morbidity. The pathogenesis of human disease varies with the particular *Bunyavirus*; some cause fever and rash; others encephalitis; and still others hemorrhagic disease which is sometimes associated with a renal syndrome. The reference strains of two murine group C viruses of the *Bunyavirus* genus, Caraparu and Oriboca, are hepatotropic in mice (Shope, 1985). We have developed a stringent model for the therapeutic evaluation of immunomodulators and antiviral drugs against hepatic infection using peripheral infection of adult mice with Caraparu virus. Caraparu virus, which induces a highly reproducible hepatitis in adult mice, is a very useful model for evaluating the efficacy of drugs that affect the immune system. The only other available *Bunyavirus* model systems of hepatic disease are Punta Toro virus in weanling mice (Sidwell et al., 1990) and Rift Valley fever virus (RVFV) in adult mice (Kende et al., 1987).

Ribavirin has previously been shown to have antiviral activity against both in vitro and in vivo experimental infections with RNA viruses, including several *Bunyaviruses* (Canonico et al., 1984). For example, ribavirin was efficacious against infections with RVFV in rodents and primates (Kende et al., 1988), Punta Toro virus in rodents (Huggins et al., 1984) and Hantaan virus in rodents (Huggins et al., 1986). The efficacy of immunomodulators against a RVFV encephalitic infection has also been reported; Poly ICLC, CL246,738, quinolinamine and MTP-PE were effective, and synergy was demonstrated between either Poly ICLC or CL246,738 and ribavirin (Kende et al., 1987; Kende et al., 1988). We have previously shown that CL246,738 was not effective against a Caraparu virus liver infection (Pinto et al., 1988). Moreover, neither IFN-α nor IFN-β were effective against Caraparu virus liver infection, although IFN-γ consistently showed a low level of antiviral activity (Pinto et al., 1990). In this report, we describe the liver histopathology caused by Caraparu virus; we further describe the relative insensitivity of this model to immunomodulator-mediated antiviral protection; and demonstrate that combined treatment with IFN-γ and ribavirin can produce enhanced activity against this fulminant hepatic infection.

Materials and Methods

Mice

Virus-free, barrier-raised, 4–6-week-old female B6C3F1 mice were purchased from Taconic Farms (Germantown, NY). Mice were shipped in double-lined filter crates and housed in PLAS-LAB (Plastics Manufacture and Supplies, Lansing, MI) isolator chambers. Intercurrent infections were monitored; serum samples were periodically obtained and tested for seroconversion to mouse hepatitis virus and Sendai virus by an ELISA assay (Biocon Labs, Rockville, MD). No intercurrent infections were detected in the test mice during the

course of these studies.

Pregnant CD-1 mice were obtained from Charles River (Wilmington, MA). Suckling mice were used to prepare stock pools of Caraparu virus.

Virus

A seed stock of Caraparu virus, strain BEAN 3999 was obtained from Dr. Robert Shope (Yale Arbovirus Research Unit, New Haven, CT). 2–4-Day-old CD-1 mice were inoculated intracerebrally (IC) with 0.02 ml of virus. When moribund, the mice were killed by decapitation and their livers removed. A clarified 10% (w/v) tissue homogenate was prepared, aliquoted and stored at −70°C. Although numerous attempts were made to plaque Caraparu on a variety of cell lines, such as BHK15, BHK21 (strain 2), CER, MK_2 and NCTC, no plaques were ever observed. Caraparu virus was, therefore, titrated using a lethal dose 50% (LD_{50}) endpoint in 3–4-day-old CD-1 mice inoculated by the IC route. The titer of the virus stock was calculated to be 10^8 LD_{50}/ml. Another murine *Bunyavirus*, Oriboca, was able to plaque on all of the cell lines tested, but was not used for immunomodulator studies because it was not as efficient at causing disease in adult mice as was Caraparu.

Immunomodulators

The IFN-α used in this study was human recombinant α-A/D IFN (rHuIFN-α) obtained from Drs. M. Brunda and P. Sorter (Hoffman-La Roche, Nutley, NJ). The IFN-β was murine recombinant β-IFN (rMuIFN-β) obtained from Dr. M. Moriyama (Toray Industries, Tokyo, Japan). The IFN-γ was murine recombinant gamma IFN (rMuIFN-γ) obtained from Dr. C. Czarniecki, (Genentech, South San Francisco, CA). The IFN's were administered in a vehicle of Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) containing 0.2% of low endotoxin bovine serum albumin (BSA; Sigma, St. Louis, MO).

Ribavirin, obtained from USAMRIID, Fort Detrick, MD, was dissolved in PBS and injected intraperitoneally (IP). The mismatched polyribonucleotide – Ampligen – was supplied to USAMRIID by Dr. Paul Ts'o (The Johns Hopkins University, Baltimore, MD) or obtained from Dr. Howard Hubbell (HEM Research, Philadelphia, PA). Ampligen was dissolved in distilled water, heated to 60°C and stored at −20°C. Poly ICLC, provided to USAMRIID by Dr. Hilton Levy (NCI), was diluted in physiological saline and then injected IP. The pyrimidinone, ABMP, was obtained from Dr. Harold Renis (Upjohn, Kalamazoo, MI). It was suspended in 1% carboxymethylcellulose and vortexed to ensure uniform suspension prior to IP inoculation. *C. parvum* was obtained from Burroughs Wellcome, (Research Triangle Park, NC), and was diluted in physiological saline and injected IP.

Enzyme assays

Plasma samples were obtained from the retro-orbital sinus of control and infected mice with a heparinized Natelson capillary tube (Fisher Scientific,

Pittsburgh, PA). As an indication of the extent of liver damage, plasma levels of glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH) were measured prior to and daily after infection of B6C3F1 mice with Caraparu virus. Because of the small volume of plasma obtained from individual mice (0.02–0.05 ml per mouse) plasma samples from 3 animals were pooled prior to assaying the levels of the three enzymes. SGPT and SGOT levels were determined using a kit from Sigma Chemical (St. Louis, MO). LDH was measured in a coupled reaction with nicotinamide-adenine-dinucleotide using sodium pyruvate as the substrate (Brinton, 1982). Enzyme activities were converted to international units.

Interferon assays

The specific activities of IFN preparations were determined by measuring protection from cytopathic effects in an in vitro assay using encephalomyocarditis (EMC) virus and mouse L-929 cells in a modified microplate assay (Havell and Vilcek, 1972). Data obtained were corrected against the NIH IFN-α/β reference standard.

For IFN treatment experiments, an estimated dose of 10000 U/mouse was prepared. Each IFN preparation was titrated every time it was used in mice. From the results obtained, successive running mean titers were calculated. The running mean titer at any given time was used to determine the dilution of IFN to be used for the next animal study. However, the amount of IFN actually used in the study was always assayed and the actual titer was then used to calculate a new running mean titer. The calculated amount of IFN is given for each of the experiments presented in the tables.

Histopathology

Groups of 2 to 3 animals were anesthetized with metaphane and perfused first with phosphate-buffered saline and then with periodate-lysine-paraformaldehyde (PLP; McLean et al., 1974). The livers and brains were removed and further fixed by immersion in PLP at 4°C. After 1 h, the tissues were cut into 0.5 cm pieces and then allowed to fix overnight at 4°C. The tissue was then dehydrated, embedded in paraffin, stained with hematoxylin and eosin and examined by light microscopy.

Thin section electron microscopy

Livers were removed from adult mice killed 1–3 days after infection with Caraparu virus. The liver tissue was cut into pieces of approx. 1 mm^3 and fixed in 2.5% gluteraldehyde-0.1 M cacodylate buffer (pH 7.3) containing 50 mM L-lysine and 1% tannic acid for 2–3 h at room temperature (Lo, 1988). The liver tissue was then post-fixed in 1% aqueous OsO_4 for 1 h at room temperature, stained en bloc with 0.5% uranyl acetate in 0.15 M NaCl overnight at 4°C, dehydrated with graded ethanol and propylene oxide, and embedded in Polybed 812 resin (Polysciences, PA). Thick sections (1 μm) were stained with 1% toluidine blue and examined with a light microscope. Areas of interest were

trimmed and thin sections (80 nm) were cut with a diamond knife. The thin sections were stained first with 5% aqueous uranyl acetate for 30 min at 37°C and then with Reynold's lead citrate and examined in a JEOL 1200 EX electron microscope.

Antiviral protection studies

Experimental groups consisted of ten 4–6-week-old B6C3F1 female mice. An LD_{50} endpoint titration of the virus inoculum was included as part of each experiment to ensure that the appropriate LD_{50} was achieved. The doses, routes and treatment schedules used are specified in the appropriate results sections. Virus-infected mice were monitored daily for signs of morbidity. After 14 days, the percent mortality and median survival times (MST) of all control and experimental groups were calculated.

Statistical analysis

Statistically significant differences ($P < 0.05$) in mortality were determined by the chi-square test. The median survival time (MST) was calculated and the survival distribution data were analyzed using the life-tables method and the Lee-Desu method of group comparisons on a SPSSX package on a VAX (Lee and Desu, 1972). This procedure allows analysis of survival data with censored observations (i.e., mice still alive at the end of the observation period).

Results

Caraparu disease model

4–6-week-old B6C3F1 female mice were injected IP with 0.1 ml of serial 10-fold dilutions of Caraparu virus. Mice receiving a lethal dose of virus died on the 4th or 5th day after infection. No clinical symptoms were apparent until a few hours prior to death, when mice appeared lethargic and felt cold to the touch. In subsequent experiments, whenever possible, mice were killed when they first showed symptoms. The majority of mice receiving a sublethal dose showed no symptoms. However, a few mice infected with sublethal doses of the virus showed significant weight loss, beginning 7 days after infection. These mice did not die during the 21 day experimental observation period. A 10-fold dilution of the Caraparu pool was found to contain an LD_{90} dose of the virus. This amount was used in all subsequent antiviral drug and immunomodulator experiments.

Plasma virus titers, enzyme levels, and interferon titers

In order to obtain a more precise understanding of the Caraparu infection model, the kinetics of virus replication in vivo were monitored. Adult B6C3F1 mice were infected by the IP route with one LD_{90} dose of Caraparu. Blood samples (0.15 ml) were collected daily from the retro-orbital sinus. The plasma pooled from three mice was serially diluted 10-fold and each dilution was

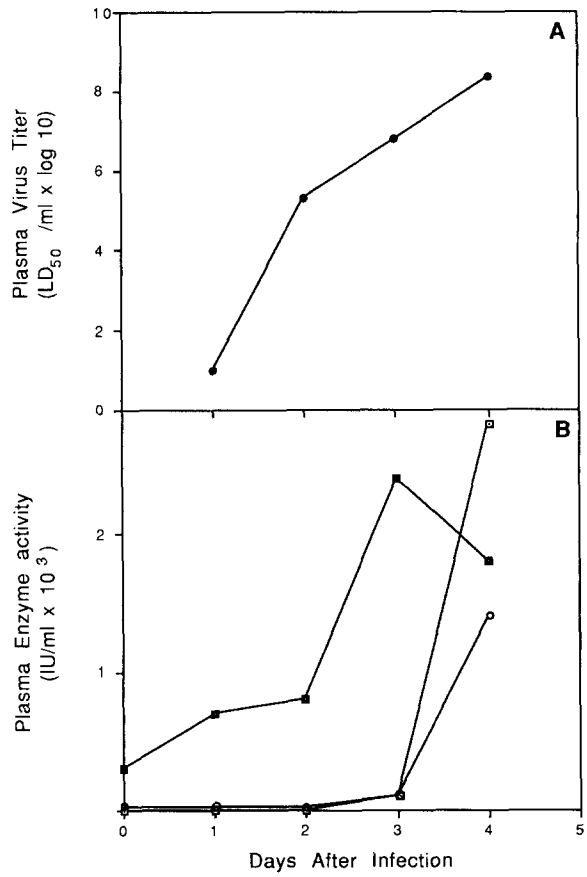

Fig. 1. Elevation of virus and enzyme levels in the plasma of adult B6C3F1 female mice with time after IP infection with Caraparu virus. (A). Virus amount (LD_{50}/ml) as determined by titration in 3-day-old mice. (B). Plasma levels of LDH (■), GPT (□) and GOT (○). Values shown were obtained from plasma samples pooled from three animals. Control enzyme levels obtained from plasma samples taken just prior to infection are plotted at the zero time point.

injected IC into a separate litter of 2–3 day-old suckling CD-1 mice (5 to 10 animals per litter). The LD_{50} virus titers obtained indicated that viremia could first be detected on day 2, and that virus titers increased each day thereafter (Fig. 1A). The plasma levels of GPT, GOT and LDH were also measured daily after infection as a means of estimating the extent of liver damage. Uninfected mice were used to obtain control enzyme levels (day 0 value, Fig. 1B). Slight increases in the levels of GPT and GOT, and a significant increase in the LDH levels were observed on days 1 and 2 after infection. By day 3, the levels of all three enzymes had increased. The level of LDH rose dramatically on day 3, while the rapid increase in the levels of GOT and GPT lagged by a day.

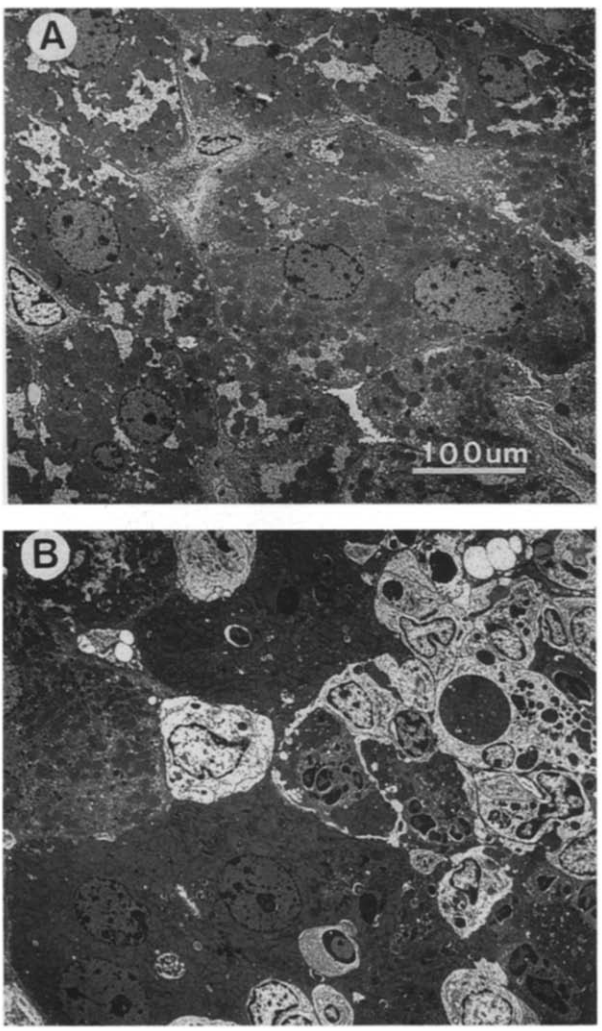

Fig. 2. Histopathology in liver tissue. (A) On days 1 and 2 after infection, liver tissue appeared normal. (B) On day 3, focal areas of necrosis were observed in 1/2 to 3/4 of the thin sections examined. Necrosis was characterized by coagulation of the hepatocyte cytoplasm and disappearance of the cytoplasmic membranes. Shrunken necrotic cells at the top of the panel contrast to normal hepatocytes in the bottom left hand side of the panel. The areas of necrosis increased in size and quantity by day 4 (data not shown). Magnification: × 1500.

Histopathology

Gross observation of organs obtained from Caraparu-infected adult mice, killed on days 1 to 4 after infection, revealed a transient enlargement of the spleen on day 1. By day 2, the spleen again appeared normal in size. The liver

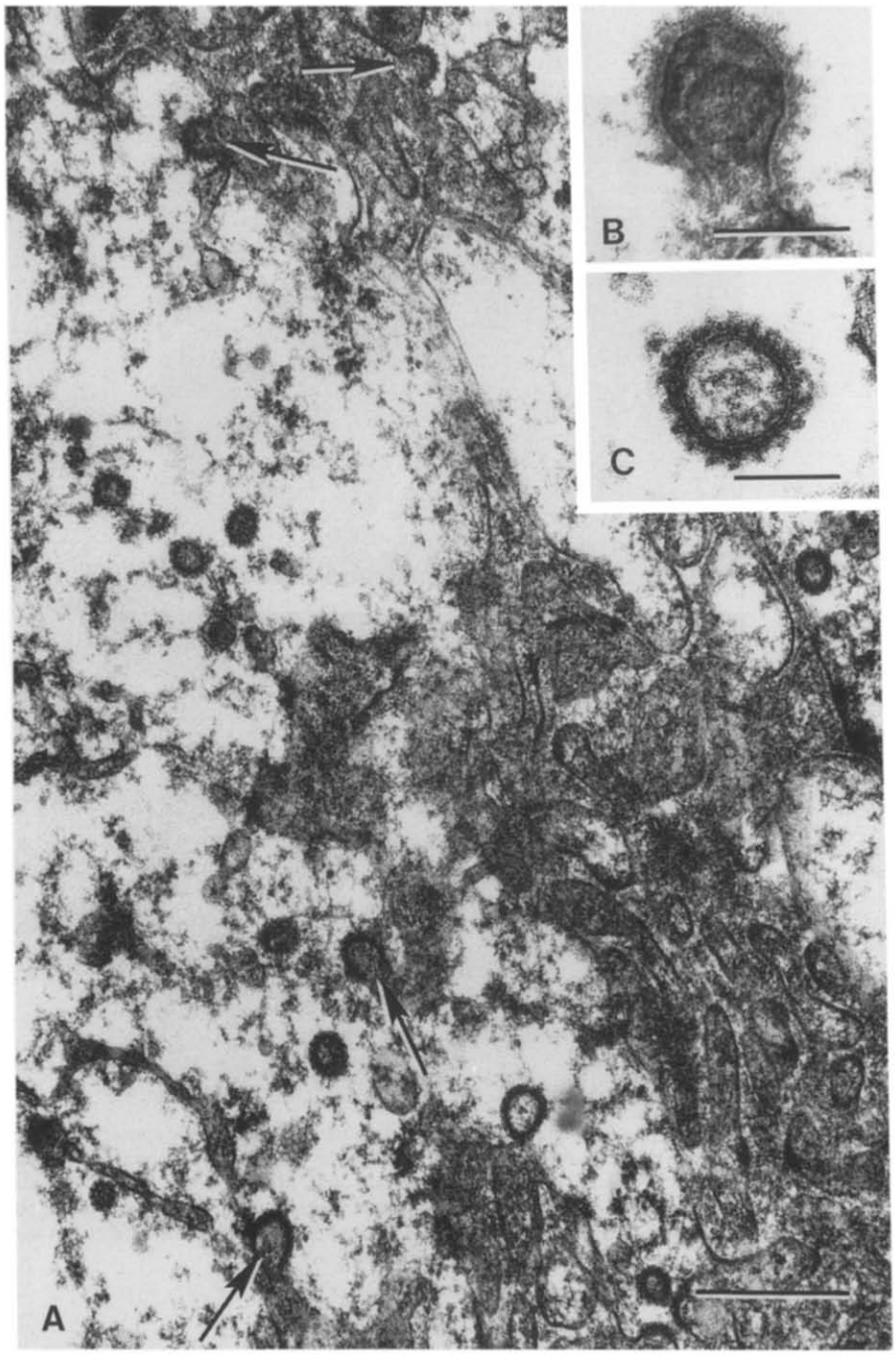

Fig. 3. Electron microscopy of Caraparu-infected liver tissue. (A) Caraparu virions in a necrotic cell. Virions in the process of budding are indicated by arrows. (B) A higher magnification of a budding virus particle. In this particle the viral nucleocapsid is visible. (C) A higher magnification of a complete virus particle clearly showing the repeating surface projections. In panel A the length of the bar is equivalent to 0.5 μm. In panels B and C, the bars are equivalent to 0.1 μm.

became noticeably pale beginning on day 3 after infection. Formalin-fixed thin sections of liver tissue were stained with hematoxylin and eosin and examined with a light microscope. On the first day after infection, the livers appeared normal. A few small foci of inflammation were observed, but similar foci were also observed in uninfected, control mice. No increase in the number of inflammatory cells was observed in liver sections on days 1 to 4 after infection. Livers from infected mice appeared normal on day 2 after infection, but by day 3, all infected livers displayed areas of coagulation necrosis. At this time, approx. one-half to three-quarters of the microscopic fields examined contained areas of focal necrosis. By day 4, massive necrosis was observed, with almost 100% of the liver tissue involved. Brain tissue sections, obtained at daily intervals after infection, were completely normal (data not shown).

Infected liver tissue was also examined by electron microscopy. Necrosis was characterized by coagulation of the hepatocyte cytoplasm. Cells in lesions were visibly shrunken and contained few cytoplasmic organelles (Fig. 2). These cells showed a general disappearance of electron dense material as well as disruption of the nuclear and cytoplasmic membranes.

Virus replication in hepatocytes

Hepatocytes in liver tissue, obtained from Caraparu-infected mice 2 days after infection, were examined by electron microscopy for the presence of virus particles. The virion size (80–100 mm in diameter) and the cytoplasmic location of intracellular virions as well as the morphology of the virus particles observed were identical to those previously reported for *Bunyaviruses* (Fenner et al., 1974; Bishop, 1985). Mature Caraparu virus particles were observed in the cytoplasm of hepatocytes in various stages of necrosis. Only a few virus particles were found in any given section of the infected cells observed. Virions were sometimes observed to be budding from the cytoplasmic membranes into intracellular vesicles. This phenomenon was observed only in cells located around the periphery of necrotic lesions, which still contained relatively normal looking cytoplasm. In cells showing necrosis, greater numbers of virus particles were observed and virions were seen budding from disrupted cytoplasmic membranes (Fig. 3). Vesicles containing virus particles were observed in Kupffer cells located within or adjacent to the liver lesions.

Immunomodulator activity against Caraparu virus infection

Caraparu virus infection was considerably less sensitive to treatment with immunomodulators than were either *Alpha Togavirus* or *Flavivirus* infections. For most of the drugs tested, the doses and schedules that were completely effective against *Alpha Togaviruses* and *Flaviviruses* (Pinto et al., 1988) were ineffective against Caraparu virus. Only two immunomodulators, poly ICLC and *C. parvum*, gave excellent protection against Caraparu virus infection as well as against *Alpha Togavirus* and *Flavivirus* infections (Pinto et al., 1988) (Table 1). Three additional synthetic immunomodulators, Ampligen, maleic anhydride-divinyl ether (MVE-2) and 2-amino-5-bromo-6-methyl-4(3H)-pyr-

TABLE 1

Effect of immunomodulators against Caraparu *Bunyavirus* infection

Drug	Dose	Schedule (days)	Mortality (%)	MST (days)
Synthetic Immunomodulators				
Ampligen	4 mg/kg	−1 to +6	100	5.7
Placebo		−1 to +6	90	5.2
Ampligen	4 mg/kg	−1 to +6	70	6.6
Placebo		−1 to +6	87	5.4
Ampligen	4 mg/kg	−1 to +6	80	8.6*
Placebo		−1 to +6	80	4.7
Poly ICLC	1 mg/kg	−1 to +6	10	>14.0*
Placebo		−1 to +6	87	4.4
Poly ICLC	1 mg/kg	−1 to +6	10	>14.0*
Placebo		−1 to +6	80	4.7
ABMP	200 mg/kg	−1	100	5.1
Placebo		−1	100	5.2
MVE-2	50 mg/kg	−1	60*	7.7*
Placebo		−1	100	5.4
MVE-2	50 mg/kg	−1	50	>19.0*
Placebo		−1	80	5.6
MVE-2	50 mg/kg	−1	20	>19.0*
Placebo		−1	73	5.0
Microbial Derived Biologicals				
C. parvum	50 mg/kg	−7	11	>19.0*
Placebo		−7	80	5.6
Recombinant Derived Biologicals				
IFN-α	10 500 U	−1 to +6	80	5.7*
Placebo		−1 to +6	90	5.2
IFN-α	10 500 U	−1 to +6	90	6.6*
Placebo		−1 to +6	100	5.4
IFN-β	7200 U	−1 to +6	100	6.6*
Placebo		−1 to +6	100	5.4
IFN-β	7200 U	−1 to +6	70	6.7
Placebo		−1 to +6	100	6.5
IFN-γ	8900 U	−1 to +6	50	>14.0*
Placebo		−1 to +6	90	5.2
IFN-γ	8900 U	−1 to +6	60	6.7*
Placebo		−1 to +6	100	5.4
IFN-γ	8900 U	−1 to +6	50	>14.0*
Placebo		−1 to +6	73	5.7

B6C3F1 female mice, aged 5–6 weeks, were injected by the IP route with the compounds and dosing schedules indicated. On day 0, all mice were infected with Caraparu virus. The mortality and MST for each individual experiment is shown. *Statistically significant ($P < 0.05$) as compared with the corresponding placebo control group.

imidinone (ABMP), which were very effective against *Alpha Togaviruses* and *Flavivirus* infections (Pinto et al., 1988; data not shown), were only partially effective or not effective, respectively, against Caraparu virus infection.

rHuIFN-α and rMuIFN-β were ineffective against Caraparu virus in prophylactic/therapeutic treatment schedules (Table 1). In contrast, a previous study indicated that regimens of prophylactic/therapeutic treatment with either rHuIFN-α, rMuIFN-β or rMuIFN-γ were highly effective against *Alpha Togavirus* and *Flavivirus* infections (Pinto et al., 1990). Only rMuIFN-γ showed

TABLE 2

Combination treatment against Caraparu virus

Drug	Dose	Initiation (days)	Mortality (%)	MST (days)
Ribavirin	100 mg/kg	0	0*	>19.0*
Placebo		0	90	5.7
Ribavirin	100 mg/kg	0	0*	>19.0*
Placebo		0	93	5.4
Ribavirin	100 mg/kg	+1	0*	>19.0*
Placebo		+1	93	5.4
Ribavirin	100 mg/kg	+2	100	6.7*
Placebo		+2	93	5.4
Ribavirin	100 mg/kg	+2	70	6.8
Placebo		+2	100	5.4
Ribavirin	100 mg/kg	+2	100	5.4*
Placebo		+2	100	4.5
IFN-γ	500 U	0	100	5.0
Placebo		0	100	5.4
IFN-γ	500 U	0	100	5.2*
Placebo		0	100	4.5
IFN-γ	500 U	+2	100	4.8*
Placebo		0	100	4.5
Ribavirin +	100 mg/kg	+2	30*	>19.0*
IFN-γ	500 U	0		
Placebo			100	5.4
Ribavirin +	100 mg/kg	+2	40*	>19.0*
IFN-γ	500 U	0		
Placebo			100	4.5
Ribavirin +	100 mg/kg	+2	90	5.6*
IFN-γ	500 U	+2		
Placebo			100	4.5
Ribavirin +	100 mg/kg	+2	70	6.3*
IFN-γ	1000 U	+2		
Placebo			100	5.3

B6C3F1 female mice, aged 5–6 weeks, were injected by the IP route with ribavirin and/or rMuIFN-γ beginning on the indicated day. Ribavirin treatment was given daily until day 6 after infection, and then every other day thereafter until day 16. rMuIFN-γ treatment continued until day 6 or 8 after infection. On day 0, all mice were infected with Caraparu virus. The mortality and MST for each individual experiment is shown. *Statistically significant ($P < 0.05$) as compared with the corresponding placebo control group.

consistent antiviral activity against Caraparu, although the level of antiviral activity obtained with rMuIFN-γ against Caraparu was much lower than that obtained against either *Alpha Togaviruses* or *Flaviviruses* (Pinto et al., 1990).

Antiviral efficacy of combination therapy against Caraparu infection

Ribavirin had been previously shown to have antiviral activity against several *Bunyaviruses*, such as Rift Valley Fever virus, Punta Toro virus, and Hantaan virus (Canonico et al., 1984). Ribavirin was also found to be highly effective against Caraparu virus infections in repeated treatment schedules beginning on either day −1 (data not shown), day 0 or day +1 (Table 2). However, when the initiation of treatment was delayed until day +2, most of

the antiviral activity was lost.

rMuIFN-γ consistently increased the MST when daily treatment was initiated on day -1 before Caraparu infection and was continued until day 6 (Table 1). The antiviral activity of rMuIFN-γ was lost when the initiation of treatment was delayed until day 0 or day $+2$ after infection.

Combined treatment with ribavirin and rMuIFN-γ clearly gave enhanced antiviral activity against Caraparu virus infections when rMuIFN-γ (500 U) was administered daily from day 0 to day $+6$ after infection and ribavirin (100 mg/kg) was administered daily from day $+2$ to day $+6$ and also on days $+8$, $+10$, $+12$, $+14$, and $+16$ (Table 2). There was both a significant decrease in mortality and a significant increase in MST. Given separately at these doses and schedules, neither compound was effective. There was only a slight, but statistically significant protection when rMuIFN-γ administration was also delayed until day $+2$ (Table 2). An increase in MST of one day was observed with administration of either 500 or 1000 U of rMuIFN-γ combined with ribavirin. By day $+2$, Caraparu had already reached a titer of 10^5 LD50/ml in the blood (Fig. 1). Liver damage was first apparent on day 3 (Fig. 2).

A variety of other combination treatments were also tested against Caraparu virus infection. These included combinations of: (1) Ampligen (4 mg/kg from day 0 to $+6$) and rMuIFN-γ (500 U from day 0 to $+6$); (2) Ribavirin (100 mg/kg from day $+2$ to $+8$ and also on days $+10$, $+12$ and $+14$) and Poly ICLC (1 mg/kg from day $+2$ to $+8$); (3) Poly ICLC (1 mg/kg from day 0 to $+6$ or from day $+2$ to $+8$) and rMuIFN-y (500 U from day 0 to $+6$ or from day $+2$ to $+8$). None of these combinations produced increased activity against Caraparu virus infections (data not shown).

Discussion

Caraparu infection in adult mice provides a new and highly reproducible model system of virus-induced hepatic disease. Models of hepatic disease have previously been developed in mice with two other *Bunyaviruses*, Punta Toro (Sidwell et al., 1990) and RVFV (Kende et al., 1987). Each of these models has some disadvantages that may restrict its usefulness for particular types of studies. Although RVFV induces a highly reproducible hepatitis in adult mice, it always induces a concomitant fatal encephalitis which could affect the interpretation of experimental data. In addition, RVFV is dangerous for man and can only be worked on under class III containment conditions. Punta Toro causes a fulminant hepatitis only in 3-week-old susceptible mice. The disadvantage of this age-restricted susceptibility, especially for immunomodulator experiments, is that the immune system of the test animals is rapidly changing due to maturation during the experimental period. Caraparu provides a particularly useful hepatitis disease model for studying both antiviral chemotherapy and immunotherapy agents. Although newborn mice (2–4 days old) develop hepatitis and encephalitis after intracerebral injection of Caraparu

virus, adult mice inoculated with virus by the IP route develop only hepatic disease. The rapid onset of death (4–5 days) in adult mice infected with Caraparu virus provides a stringent model for testing the efficacy of therapeutic agents. A disadvantage of the Caraparu model is that, since this virus does not induce plaques in tissue culture, virus must be titered in animals. Two additional models of hepatitis caused by arthropod-borne viruses, yellow fever virus in primates (Monath et al., 1981) and Pichinde in guinea pigs (Lucia et al., 1990) each have the disadvantage of utilizing larger, more expensive test animals and having a longer disease course.

Caraparu-infected mice do not display any clinical symptoms until a few hours prior to their death. This is similar to what is observed in guinea pigs infected with Pichinde virus (Lucia et al., 1990). The titer of virus in the plasma of Caraparu virus infected mice was higher than that observed during infections with other types of *Bunyaviruses* in mice. The fact that Caraparu is a murine *Bunyavirus* may explain its efficient growth in mice. The rapid increase in the plasma titers of Caraparu in infected mice, parallels the increase in virus-infected hepatocytes in the liver with time after infection.

Although the Caraparu virus hepatic infection model was the least sensitive to immunomodulator treatment, as compared with *Alpha Togavirus, Flavivirus* and herpes simplex virus infections (Pinto et al., 1988), it is noteworthy that some immunomodulators, such as *C. parvum*, MVE-2 and Poly ICLC did show substantial activity against Caraparu virus. The effectiveness of MVE-2 against Caraparu virus further supports the use of this type of polyanion as a broad spectrum antiviral agent. The activity of MVE-2 appears to involve multiple mechanisms such as IFN induction, and macrophage and NK cell activation. (Chirigos et al., 1980, Morahan et al., 1986; Wiltrout et al., 1984). Poly ICLC, but not Ampligen, was effective against Caraparu virus infection even though both compounds are synthetic polyribonucleotides that induce IFN α/β (Hubble, 1986; Kende et al., 1987). The reasons for the observed differences in the efficacies of different polyribonucleotides have not yet been delineated. Interestingly, treatment of mice with high titer antibody to IFN-α/β partially reduced the antiviral activity of Ampligen, but did not affect the activity of Poly ICLC, against Semliki forest virus infection (Morahan et al., 1991). Moreover, treatment of Caraparu-infected animals with IFN-α and -β was ineffective (Table 1). Together, these data suggest that Poly ICLC, but not Ampligen, may induce Caraparu antiviral activity through additional mechanisms not associated with IFN-α/β, and/or through local induction in the liver of interferon or other antiviral factors.

Although neither rHuIFN-α nor rMuIFN-β gave significant protection against Caraparu infection, rMuIFN-γ showed a modest, but consistent activity against Caraparu in both repeated prophylactic/therapeutic and repeated early therapeutic treatment schedules (Table 1; Pinto et al., 1990). The reason for the antiviral efficacy of gamma IFN-γ has not yet been explained. Several previous studies have shown that IFN-γ is a more powerful macrophage activator than either IFN-α or IFN-β (Murray et al., 1985;

Murray, 1988; Varesio et al., 1984; Wilson et al., 1985). Moreover, although all three types of IFN can augment NK cell activity (Biron and Welsh, 1982; Daurat et al., 1988; Pinto et al., 1988), IFN-γ has been found to activate NK cells by a different mechanism than IFN-α or IFN-β (Gustafsson et al., 1986; McGinnes et al., 1988). The three IFN's, then, may induce macrophages and NK cell populations with different antiviral states or effector functions. Since IFN-γ is such a potent activator of macrophages and NK cells, it may induce a particularly effective early defense mechanism in the liver macrophages and NK cells of Caraparu-infected mice (Morahan and Murasko, 1988). A number of investigators have shown that IFN-γ has potent antimicrobial activity against infections with liver involvement, such as *Candida albicans* (Garner et al., 1989), *Listeria monocytogenes* (Kurtz et al., 1989; Kiderlen et al., 1984), *Rickettsia conorii* (Li et al., 1987) and *Plasmodium berghei* (Ferreira et al., 1986). IFN-γ was much more effective than was IFN-α/β in inhibiting the development of *Plasmodium falciparum* in human hepatocyte cultures (Mellouk et al., 1987). The possible differences in the actions of the IFN's in liver Kupffer cells and hepatocytes merit additional investigation.

The present results establish the efficacy of ribavirin against fulminate hepatic *Bunyavirus* infections. Ribavirin, a synthetic nucleoside with an incomplete purine ring, has antiviral activity against a broad spectrum of RNA viruses (Sidwell, 1980). Several *Bunyavirus* infections have clearly been shown to be very responsive to therapeutic treatment in vivo with ribavirin (Canonico et al., 1984; Kende et al., 1987; Sidwell et al., 1988).

Combination therapy with two antiviral agents, each with a different mechanism of antiviral action, is of interest because it may permit the use of smaller drug doses and also allow delay of the initiation of treatment. The present results are the first, to our knowledge, that demonstrate enhanced activity with IFN-γ and an antiviral drug. Several previous studies have shown that recombinant IFN-α can give synergistic activity with an antiviral drug. Enhanced efficacy of the nucleoside analog acyclovir [9-(2-hydroxy-ethoxymethyl) guanine] against herpes simplex virus type 1 (Connell et al., 1985) and of the acyclic nucleoside DHPG [9-(1,3-dehydroxy-2-propoxymethyl) guanine], vidarabine, or vidarabine 5'-monophosphate against herpes simplex virus type 2 (Fraser-Smith et al., 1984; Crane and Sunstrum, 1988) was observed in mice when these drugs were given in combination with rHuIFN-α. Synergism between IFN-α and either rimantadine or ribavirin was observed in vitro against influenza A virus (Hayden, 1986). Kende et al. (1987, 1988) have shown synergistic action against RVFV upon combination treatment with ribavirin and either Poly ICLC or CL246,738, but not quinilonamine. Our results indicate that enhanced efficacy can also be obtained with combination therapy utilizing IFN-γ and an antiviral drug, ribavirin, in vivo. Because IFN-γ is a potent activator of macrophages and NK cells (Pinto et al., 1988), it may be the best IFN for combination therapy against viruses which cause a rapid visceral pathology.

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