



## Ribavirin efficacy in an *in vivo* model of Crimean-Congo hemorrhagic fever virus (CCHF) infection<sup>1</sup>

Gregory H. Tignor\* and Catherine A. Hanham

*Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University  
School of Medicine, New Haven, CT 06510 (USA)*

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### Summary

After intraperitoneal (i.p.) infection of infant mice with CCHF virus, virus titers in liver remained significantly higher than in other organs except blood (serum). Within the liver, virus antigen was first found by immunofluorescence (IFA) in Kupffer cells followed by more extensive hepatic spread. Later, virus was found in other organs including brain and heart. Ribavirin treatment significantly reduced infant mouse mortality and extended the geometric mean time to death. Ribavirin treatment reduced CCHF virus growth in liver and significantly decreased, but did not prevent, viremia. Despite a substantial viremia, infection of other organs including brain and heart was not detected in ribavirin-treated mice. A hepatotropic virus subpopulation with less neurovirulence than the parent was isolated from liver of ribavirin-treated mice (single dose, 100 mg/kg). After serial passage in placebo-treated mice, the exclusive hepatotropism was lost.

Ribavirin; Crimean-Congo hemorrhagic fever, CCHF; Virus infection

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### Introduction

Infection of humans with tick-borne Crimean-Congo hemorrhagic fever virus (CCHF) often results in a serious illness followed by death. It has been estimated that one-third of hospitalized CCHF patients die although many

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\*Corresponding author. Fax: +1 (203) 785 4782.

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recover rapidly after a febrile illness (Gear, 1988). Cases that develop after person-to-person transmission via contaminated blood are often particularly severe. Although clinical indicators of severity have been developed, there is no understanding of why the illness is severe in some but not in others. The etiologic agent of CCHF is a single-stranded RNA virus of the *Nairovirus* genus in the Bunyaviridae family (Casals and Tignor, 1980). It is antigenically uniform throughout its range in Africa, Europe and Asia (Tignor et al., 1980). Other unusual aspects of the epidemiology of CCHF have been reviewed in detail by others (Hoogstraal, 1979; Watts et al., 1988).

CCHF vaccine is not available commercially outside Bulgaria and the Federated Commonwealth States. Administration of plasma from immune donors is considered by some to be the standard specific treatment for patients with CCHF (Gear, 1988). However, immunotherapy in CCHF is controversial and results are inconsistent. One view is that the variable results after human immunotherapy trials reflect the difficulty in getting donors with high antibody titers (Monath, 1990). Immune plasma or globulin cannot be used prophylactically because the supply is limited and neutralizing antibody titers in convalescent plasmas are low. In actual practice, only patients with a well-established CCHF diagnosis receive immune plasma. In one study, only transient improvement in clinical status was noted, without a clear effect on viremia (Van-Eeden et al., 1985).

There has been limited experience with alternative forms of CCHF therapy. During a 1984 outbreak of CCHF at Tygerberg Hospital in South Africa, two different antiviral agents, ribavirin and human leukocyte interferon, were given. Ribavirin was used prophylactically in six of nine CCHF-case contacts who sustained direct blood exposure by various means including needle-punctures. Three individuals also received a short course of interferon. Of the six treated contacts, one (who did not receive interferon) developed a mild case of CCHF. Two of three untreated contacts developed severe CCHF. Interpretation is clouded by the fact that one untreated contact and an additional 42 other untreated individuals who had contact with contaminated blood did not develop disease (Van-de-Wal et al., 1985). South African patients who were treated with high doses of human leukocyte interferon either prophylactically or therapeutically (together with plasma) developed severe side-effects; the practice was stopped (Van-de-Wal et al., 1985).

Six years later, a brief report was given of the result of treating 12 CCHF patients with ribavirin in open-label trials in South Africa (Swanepoel et al., 1990). When ribavirin therapy was started early (4 days post-onset), none of the seven patients died. However, of the seven survivors, only two had clinical laboratory markers associated with >90% mortality in historical controls. After late initiation of therapy (5 days post-onset), three of the five patients died.

The purpose of this research was to study the effect of ribavirin in a laboratory model of CCHF virus infection. Outbreaks of CCHF have been unpredictably sudden, frequent enough, and of enough seriousness to warrant antiviral drug study. However, laboratory animal models for CCHF infection

have been difficult to establish because frequently used laboratory animals show little, if any, sign of infection or disease (Gear, 1988). Also, laboratory work with CCHF has been limited because accidental infections that have had serious or even fatal outcomes have too frequently occurred (C.D.C., 1984). As a result, little is known about the pathogenetic mechanisms governing the expression of human disease in CCHF infection. So, as has been done in similar situations, both the candidate compound, ribavirin, and the experimental model itself were examined in this study (Grunert, 1979). The experimental model used in these experiments was an infant mouse model based on intraperitoneal (i.p.) inoculation of CCHF virus.

## Materials and Methods

### *Ribavirin testing in the in vivo model of CCHF virus infection*

Ribavirin (1- $\beta$ -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was supplied by Dr. John Huggins, Department of Antiviral Studies, Virology Division, US Army Medical Research Institute of Infectious Diseases, Frederick, Maryland. Either a 50 or 100 mg/kg dose was inoculated intraperitoneally (i.p.) into infant mice in a volume of 0.075 ml. 45 min later, virus (50–200 LD<sub>50</sub>) was inoculated i.p. in a volume of 0.075 ml. The virus was passaged to level 8 in infant mouse tissue of CCHF strain IbAr 10200 isolated in Nigeria, and described previously (Tignor et al., 1980). Placebo-treated mice were inoculated with Dulbecco's minimal essential medium (DMEM), the diluent for the drug. The virus was titrated simultaneously. In multiple drug dose tests, a total of four more daily drug doses was given after injection of virus.

Infected mice were housed and observed daily in a biosafety level 3+ animal facility modified especially for this work. Sentinel mice were tested serologically for intercurrent murine infections by the Yale University Division of Animal Care.

### *Biologic safety*

Class 2 biological safety cabinets were used in a biological safety level 3 laboratory for manipulations involving infectious or potentially infectious materials. Individual workers were equipped with HEPA-filtered respirators and underwent serologic surveillance throughout the course of this project.

### *Analysis of data*

Geometric mean times to death were calculated for placebo-treated mice and drug-treated mice. The geometric mean time to death (GMTD) was equal to the  $n$ th root (where  $n$  = the total number of animals) of the product of each day with mortality raised to the power of the number of animals dying on that day. In this calculation, survival was defined as 21 days.

Correlations and the statistical significance of differences between drug- and placebo-treated groups were determined by standard tests as described earlier

(Tignor et al., 1980) using SYSTAT (Wilkinson, 1989).

A direct measure of ribavirin effect on mouse mortality was used as comparison with the GMTD. In this case, a log protective index (PI) was calculated. This was determined by subtracting the log LD<sub>50</sub> virus titer in drug-treated animals from the log LD<sub>50</sub> virus titer in placebo-treated animals. A log protective index of 1.7 meant at least 50% protection against 50 LD<sub>50</sub> of virus. This technique has been widely used for determination of protection mediated by virus-neutralizing antibody (Shope, 1974).

#### *Pathogenesis of CCHF in infant mice*

Placebo-treated and ribavirin-treated mice were each divided into two groups, one of which (16 mice) was held for observation. Virus was titrated daily from the other group by inoculating CER cells with blood serum, liver, brain, spleen, and heart tissue homogenates. SW-13 cells (supplied by Dr. D. Watts, US Army Medical Research Institute of Infectious Diseases) were also used in some tests. Tissues from eight animals were pooled and examined in four replicate cultures at each interval. Mice were first exsanguinated and then perfused with DMEM. Other tissues were removed, weighed, homogenized, clarified by light centrifugation and the supernatant fluid inoculated into freshly made cultures as described for earlier pathogenesis experiments (Tignor et al., 1973). Virus titers were determined by fluorescence assay of infected cells under semi-solid overlay and expressed as FFU/inoculum as described earlier (Tignor et al., 1980). The variation between individual titers was less than 0.3 log<sub>10</sub>.

Infected tissues were also examined by immunofluorescence. Tissue sections from 6 mice were cut to 4–6 µm thickness, mounted, fixed in acetone, stained with CCHF or control rabies virus antibody, washed in PBS, and stained with fluorescein-conjugated goat anti-mouse antibody (Antibodies Incorporated, Davis, CA). Co-localization of virus and specific cell types was by peroxidase staining of paraffin-embedded tissues using a commercial kit (ExtrAvidin Biotin Staining Kit, Sigma, St. Louis, MO) and virus-specific or cell-specific monoclonal or polyclonal antibodies. Mac-1 antibody (monoclonal antibody, clone M1/70HL to a surface antigen found on mouse macrophage cells) was purchased commercially (Boehringer-Mannheim Biochemicals, Indianapolis, IN). This antibody has been described as being weakly reactive with liver tissue macrophages (Ho and Springer, 1982).

#### *Serology*

Preparation and use of CCHF virus polyclonal antibody in IFA and neutralization tests was as earlier described (Tignor et al., 1980). Antibodies for immunofluorescence observation included those described in an earlier publication and others from the reference collection of the Yale Arbovirus Research Unit (Casals and Tignor, 1980): Bandia, Bwamba, California encephalitis, Caraparu, Chenuda, chikungunya, Colorado tick fever, dengue 3, Dhori, Dugbe, Germiston, Hazara, Japanese encephalitis, Kemerovo,

lymphocytic choriomeningitis, Naples sandfly fever, Oropouche, Qalyub, rabies, Sindbis, Venezuelan equine encephalitis, Wad Medani, West Nile, and yellow fever. Neutralization tests for monitoring the purity and identity of virus isolated after necropsy were performed using constant antibody and varying virus dilutions. In this protocol, mice that escaped neutralization (i.e., 'breakthrough' mice) were examined by IFA to establish the identity of the virus in the tissues.

Hybridomas producing monoclonal neutralizing anti-CCHF antibody were prepared after intrasplenic inoculation of virus using otherwise standard methods (Spitz et al., 1984; Kohler and Milstein, 1975). The monoclonal antibodies were isotyped using a commercial kit (Zymed Laboratories, San Francisco, CA). Screening was done by IFA and immunoprecipitations were carried out as described elsewhere (Hanham et al., 1993).

## Results

### *Relation between virus dose and geometric mean time to death (GMTD)*

In placebo-treated animals, there was an inverse relationship (correlation coefficient,  $r^2=0.77$ ) between the geometric mean time to death (GMTD) and CCHF virus dose (50–200 LD<sub>50</sub>). At higher virus doses, there was less correlation with GMTD, because mice were not killed less than 5 days after i.p. inoculation regardless of virus dose. Using a 50 LD<sub>50</sub> dose of virus from the same frozen virus stock in 6 tests, the GMTD ranged from 7.9 days to 8.2 days (mean  $8.0 \pm 0.6$ ).

### *Ribavirin in the CCHF model*

When given in a single dose early after infection, ribavirin was an effective treatment in doses as low as 50 mg/kg in the CCHF model. In 19 tests using an exact 50 LD<sub>50</sub> virus dose, the mean GMTD was  $15.2 \pm 0.5$  days, an increase of 7.2 days ( $P=0.0001$ ). Using a constant 50 mg/kg dose of ribavirin, a direct relationship (correlation coefficient,  $r^2=0.88$ ) existed between ribavirin GMTD and virus dose (25–200 LD<sub>50</sub>). The lowest increase in GMTD was 3.2 days ( $200 \pm 80$  LD<sub>50</sub>;  $P=0.002$ ) and the greatest, 12.0 ( $25 \pm 10$  LD<sub>50</sub>) (Table 1).

Using 50 LD<sub>50</sub> of virus, the GMTD after multiple ribavirin doses (50 mg/kg, for 5 days) was higher ( $20.4 \pm 0.3$ ) than after a single dose ( $15.2 \pm 0.5$ ). The GMTD after multiple injections of 100 mg/kg was  $21 \pm 0.4$ . However, a single 100 mg/kg dose was nearly as effective ( $20.2 \pm 0.4$ ) as multiple doses (100 mg/kg) (Table 1). Beginning treatment on day 5, at the earliest time of onset of clinical signs, had no effect on either mortality or GMTD.

The GMTD was compared to the log protective effect (PI). The PI was a direct measure of reduced mortality attributable to ribavirin treatment. The correlation coefficient ( $r^2$ ) was 0.76. An increase in GMTD of 3.2 days (range 3.1–3.5) was equivalent to a 1.7 PI.

These data were based on changes in GMTD. Experiments were then done

TABLE 1

Ribavirin treatment of Crimean-Congo hemorrhagic fever (CCHF) virus-infected infant mice

CCHF virus strain 10200 <sup>a</sup>		Ribavirin/placebo treatment <sup>b</sup>		Outcome <sup>c</sup>	
Number of tests	Virus dose (LD <sub>50</sub> )	Drug dose (mg/kg)	Drug dose schedule	Percent mortality	Geometric mean time to death
6	50 ± 15	Placebo	Single	100	8.0 ± 0.6
19	50 ± 15	50	Single	15	15.2 ± 0.5
6	50 ± 15	100	Single	2	20.2 ± 0.4
2	25 ± 10	Placebo	Single	100	8.6 ± 0.4
2	25 ± 10	50	Single	12	20.6 ± 0.4
2	100 ± 24	Placebo	Single	100	7.3 ± 0.9
2	100 ± 24	50	Single	37	14.4 ± 0.5
2	125 ± 33	Placebo	Single	100	7.6 ± 1.2
2	125 ± 33	50	Single	37	13.3 ± 0.6
2	175 ± 50	Placebo	Single	100	7.3 ± 1.6
2	175 ± 50	50	Single	50	11.0 ± 0.8
2	200 ± 80	Placebo	Single	100	7.0 ± 2.2
2	200 ± 80	50	Single	50	10.2 ± 0.8
6	50 ± 25	Placebo	Multiple	100	7.4 ± 0.2
6	50 ± 25	50	Multiple	12	20.4 ± 0.3
6	50 ± 25	100	Multiple	0	21.0 ± 0.4

<sup>a</sup>Passage 6 in infant mouse brain tissue, i.p. inoculation.<sup>b</sup>Ribavirin (single) was given i.p. 45 min after virus or multiple with four additional doses on consecutive days. The placebo was culture medium (DMEM).<sup>c</sup>8–10 mice in each test group.

to determine if increased GMTD, after ribavirin treatment was a result of decreased virus replication in a specific target organ.

#### *Pathogenesis of CCHF virus in placebo-treated and ribavirin-treated infant mice*

After i.p. CCHF virus infection (50 LD<sub>50</sub>) of infant mice, virus replication was first detected in the liver with subsequent spread to the blood (serum). Virus titers were slightly higher in the liver than in the serum (Fig. 1A) from day 3 to day 7 (last surviving day for placebo-treated mice). Relative to virus growth in liver tissue, virus appeared very late after infection in other tissues including the brain (day 7, Fig. 1A), and heart (day 6, Fig. 1B). Virus was not isolated from the spleen after day 2 (Fig. 1B). Virus titers were higher in the liver than in other organs, with the possible exception of the serum. The differences in virus titer between the liver and brain, heart and spleen were significant ( $P < 0.05$ , paired  $t$ -test). However, the differences in titer between the liver and serum were not statistically significant.

Ribavirin-treated mice (single dose, 50 mg/kg) had significantly lower virus titers in liver ( $P = 0.007$ , paired 2-tailed  $t$ -test; Fig. 2A) and a significantly reduced viremia ( $P = 0.007$ , paired 2-tailed  $t$ -test; Fig. 2B). Although virus growth in liver was suppressed by ribavirin treatment, a large viremia was

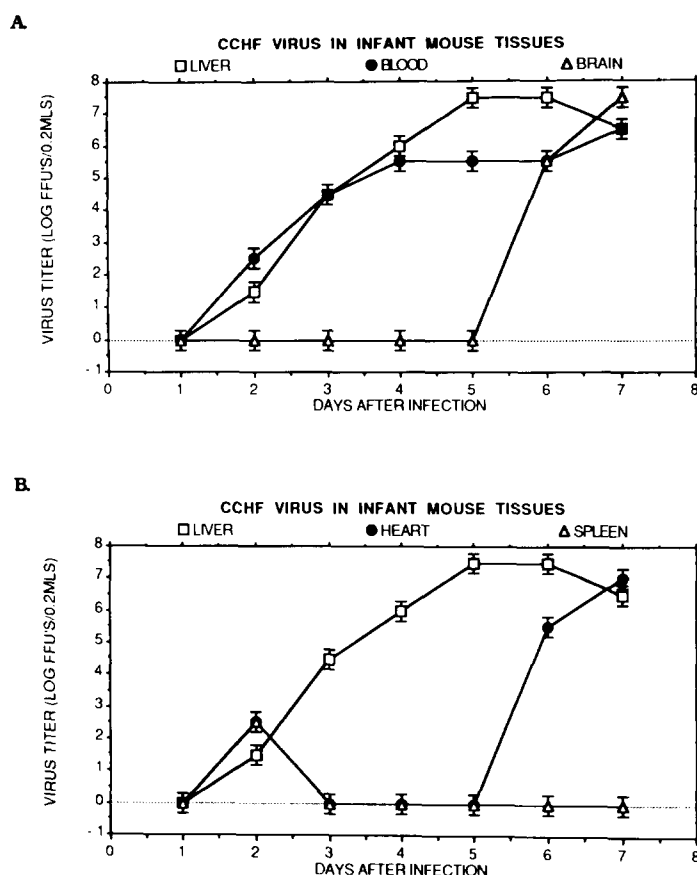


Fig. 1. Titers of CCHF virus in infant mouse tissues. (A) CCHF virus titers in liver, blood (serum) and brain of infant mice. (B) CCHF virus titers in liver, heart and spleen of infant mice.

detected beginning on day 5. Despite the viremia, virus was not isolated from the heart, spleen or brain. None of the treated mice held for observation died. In this experiment; mice were not examined after day 7.

The liver was examined by both IFA and immunoperoxidase techniques using both frozen and paraffin-embedded tissue. 3 days after inoculation of virus, CCHF virus antigen occurred in what appeared morphologically to be Kupffer cells lining the liver sinusoids of placebo-treated mice. Animals treated with a single dose of ribavirin had very little or no demonstrable CCHF antigen in these cells at this time.

Later, in placebo-treated animals, CCHF virus antigen was present in many clusters of hepatocytes widely distributed throughout liver tissue including occasional tissue macrophages. Infection of Kupffer cells was not prominent (Fig. 3A). CCHF antigen was sparsely seen in liver tissue of ribavirin-treated animals, sometimes in a putative Kupffer cell or sometimes in a hepatocyte

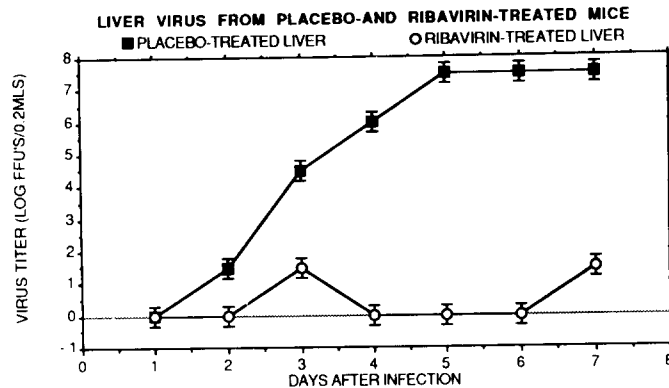
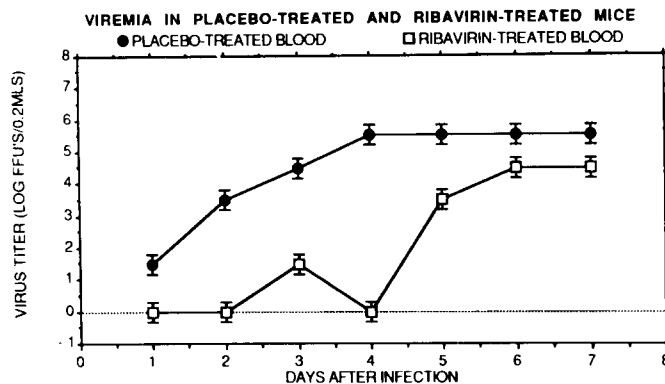
**A****B**

Fig. 2. Titers of CCHF virus in ribavirin-treated infant mice (single dose, 50 mg/kg, 45 min after virus inoculation). (A) CCHF virus titers in liver tissue from placebo-treated (DMEM) and ribavirin-treated mice. (B) Viremia in placebo-treated and ribavirin-treated mice.

(Fig. 3B). Cells similar to those in Fig. 3B were seen up to 10 days after infection even in apparently healthy mice treated with multiple doses of ribavirin (50 mg/kg). Mock-infected controls showed no evidence of infection.

Kupffer cells were identified by immunoperoxidase staining on serial sections of paraffin embedded liver tissue sections using anti-Mac-1 antibody; infected cells were identified using anti-CCHF antibody. In consecutive tissue slices, some Mac-1 antibody-positive cells, i.e., Kupffer cells (Fig. 4A), were also positive with anti-CCHF antibody on day 3 (Fig. 4B).

#### *Characteristics of CCHF virus from ribavirin-treated mice*

In the experiments described above, evidence of CCHF virus infection of the liver was found in apparently healthy ribavirin-treated mice. Despite a



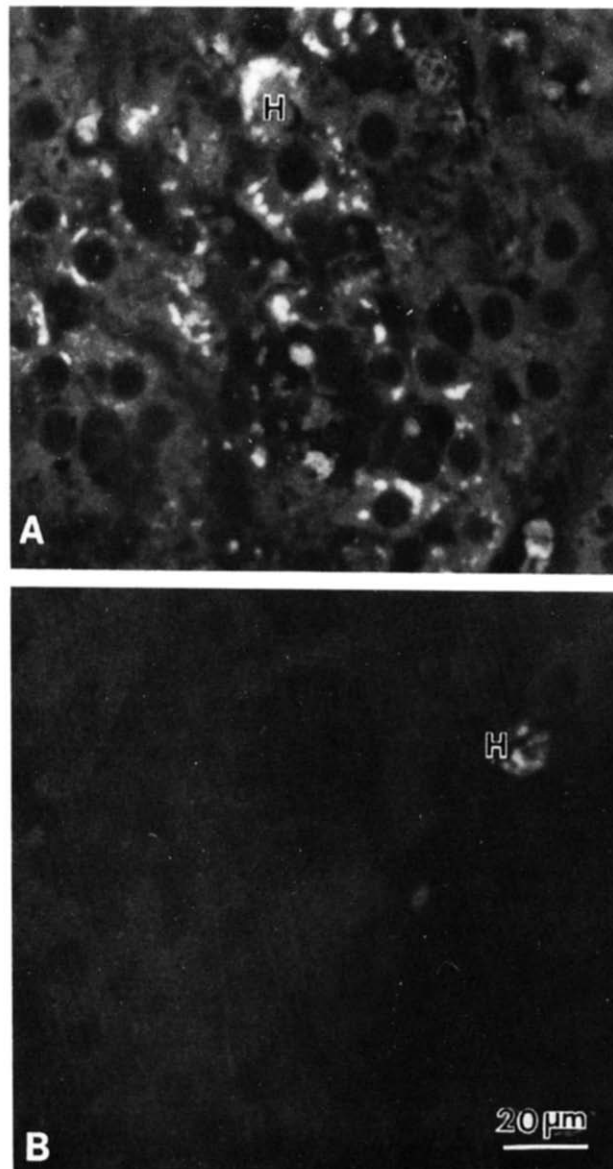


Fig. 3. The effect of a single dose of ribavirin (50 mg/kg) on the distribution of CCHF virus antigen in infant mouse liver tissue 7 days after inoculation of virus. (A) CCHF virus antigen is present in many clusters of hepatocytes (H) in the liver of an infected animal given placebo. (B) CCHF virus antigen is significantly less present in liver tissue of animals treated with ribavirin. A single infected hepatocyte (H) is shown.

relatively high viremia, infection did not spread to the brain or other organs. This result was different from that in placebo-treated animals where viremia

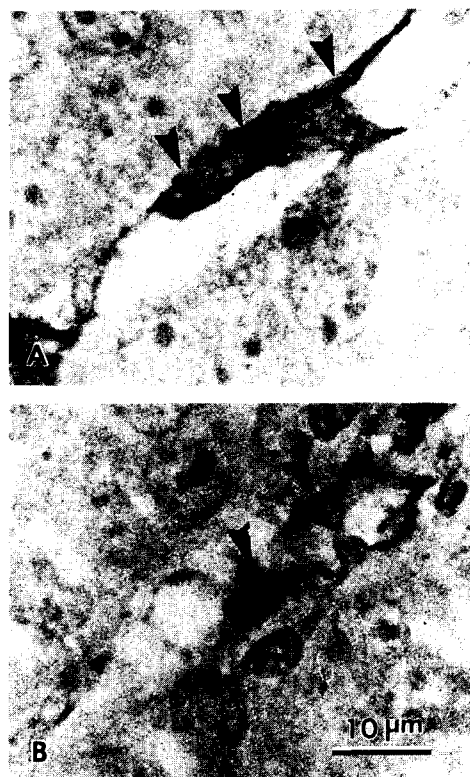


Fig. 4. Identification of CCHF virus-infected cells as Kupffer cells by immunoperoxidase staining. The distribution of Mac-1 antibody and CCHF virus antibody in Kupffer cells on the third day after infection. (A) Mac-1 antibody reactivity present in a Kupffer cell lining a liver sinusoid. (B) In a consecutive section, CCHF virus antibody reactivity occurs in the same Kupffer cell.

was followed by infection of other organs (brain and heart).

These results suggested that the protective effect of a single dose of ribavirin (50 mg/kg) diminished with time. Therefore, the experiment was repeated, focusing only on viremia in ribavirin-treated mice and using a higher ribavirin dose (100 mg/kg). Additional doses of ribavirin were given on days 5 and 9. Virus isolations were not done before day 6. Viremia ( $\log_{10}$  FFU/ml) was found on days 6 ( $3.6 \pm 0.2$ ), 7 ( $4.5 \pm 0.2$ ), 8 ( $2.6 \pm 0.2$ ), 9 ( $2.4 \pm 0.3$ ) and continued at that level through day 11. On day 12, the titer ( $\log_{10}$  FFU/ml) decreased to  $1.3 \pm 0.3$ ; virus was not found on days 13 or 14. Despite the relatively prolonged viremia, virus was isolated only from the liver (days 6–13), not from the brain, heart or spleen. Titers of liver virus were not determined. Mice held for observation did not die.

Liver tissue from CCHF virus-infected, ribavirin-treated mice (single dose, 100 mg/kg) was titrated daily by i.p. subinoculation into infant mice. The peak

titer in the liver was found on day 8. Eight ribavirin-treated mice (single dose, 100 mg/kg) without clinical signs of illness were necropsied on the eighth day after i.p. virus injection (50 LD<sub>50</sub>). Virus was found in pooled serum and supernatant fluid from tissue homogenates of pooled liver but not other organs including brain, heart, and spleen. The infectious virus titer (log<sub>10</sub> LD<sub>50</sub>/ml) in the liver was 4.0 and 2.3 in the serum as determined by i.p. inoculation of infant mice. Virus from liver tissue of ribavirin-treated mice grew very poorly in CER or SW-13 cells. For example, in individual wells of Lab-Tek slide trays containing  $5 \times 10^4$  CER cells, 2–10 positive cells were observed by IFA. Results were similar with SW-13 cells. Residual ribavirin in the tissue homogenates could have inhibited virus growth in vitro. Therefore, the virus was pelleted from the supernatant fluid by ultracentrifugation (100 000  $\times$  g, 60 min), but virus infection of cultured CER cells was not increased. Because of the variability between individual cultures in the number of infected cells, it was impossible to determine in vitro ribavirin sensitivity reliably.

Attempts were made to determine ribavirin sensitivity in vivo. The original liver tissue virus from ribavirin-treated mice was used as source in an in vivo ribavirin drug test. Mice were infected with liver virus (50 LD<sub>50</sub>, i.p.) and treated with a single dose of ribavirin at either 50 or 100 mg/kg. The GMTD in mice treated with 50 mg/kg was unchanged from the placebo controls (mortality 8/8) and the GMTD in mice treated with 100 mg/kg was increased by 1.6 days (mortality 8/8). Eight of eight (8/8) placebo-treated animals died with an GMTD of 8.1 days.

Virus isolations were done by i.p. inoculation of infant mice using pooled tissues from two mice taken on days 7 and 8 from the two groups, one treated with 100 mg/kg and one placebo-treated. Virus was isolated from the liver and serum in both treated and placebo-treated mice on days 7 and 8 but not from other organs on either day. In treated mice (100 mg/kg), virus titers (log<sub>10</sub> LD<sub>50</sub>/ml) were as follow. Day 7: liver, 4.2; serum, 2.9; day 8: liver, 4.5; serum, 2.7. Virus titers in placebo mice were as follow. Day 7: liver, 5.7; serum, 5.3; day 8: liver, 5.2; serum, 5.3. These data show that ribavirin treatment (100 mg/kg) reduced virus titers in treated animals. In contrast to an earlier experiment with the parent virus, treatment with ribavirin at 50 mg/kg had no effect on virus titers (data not shown). Despite the relatively high viremia in placebo-treated mice (5.3 log<sub>10</sub> LD<sub>50</sub>/ml), virus was not isolated from other tissues (brain, heart, spleen). This suggested a marked hepatotropism for the injected virus.

Three blind passages of the hepatotropic virus through placebo-treated mice resulted in a loss of strict hepatotropism. After three i.p. passages of liver tissue virus harvested on day 7, sick infected mice were necropsied. Isolated virus had the following titers (log<sub>10</sub> LD<sub>50</sub>/ml): liver ( $6.1 \pm 0.2$ ); serum ( $5.6 \pm 0.2$ ); brain ( $7.1 \pm 0.2$ ); heart ( $6.6 \pm 0.2$ ). Strict hepatic tropism was no longer in evidence.

#### *Identity and purity of virus*

Tissues from mice infected with liver virus from ribavirin-treated animals were examined by immunofluorescence using the battery of antiviral antibodies

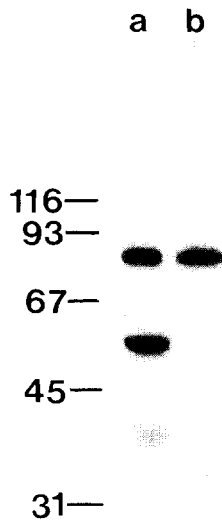


Fig. 5. Immunoprecipitation of radiolabeled ( $^{35}\text{S}$ ) CCHF virus proteins from the supernatant fluid of infected CER cell cultures. Proteins immunoprecipitated by polyclonal CCHF virus antibody are in lane A; a single protein immunoprecipitated by monoclonal CCHF antibody 6A is in lane B. The electrophoretic mobility of the protein shown in lane B was increased slightly (4 kDa) after endoglycosidase (PNGase F, 16 h, 37°C) treatment (data not shown).

listed in Materials and Methods. Antibody to mouse hepatitis virus (MHV) was also included. This virus, a common contaminant of mouse colonies, infects Kupffer cells and is unaffected by ribavirin (Ruebner and Miyai, 1962; Robins, 1986). The only positive reaction was with CCHF virus antibody. In addition, the Division of Animal Care at the Yale School of Medicine reported no serologic evidence of MHV infection in sentinel animals housed in our animal facility.

Identity and purity were further established by a mouse neutralization test using CCHF virus mouse monoclonal antibody. This monoclonal antibody (IgG 2a) reacted by immunoprecipitation with a single radiolabeled CCHF virus protein (Fig. 5) and had a neutralizing titer of 1:64 in an i.p. mouse neutralization test using 50 LD<sub>50</sub> of CCHF virus. The breakthrough neutralization test was done with a constant amount of antibody (1:2) and varying dilutions of virus ( $10^{-1}$  to  $10^{-5}$ ). There were no breakthrough mice that showed clinical signs of illness or died in the neutralization test.

## Discussion

The purpose of this research was to test ribavirin in an *in vivo* laboratory model for antiviral activity against disease caused by CCHF virus. The only available *in vivo* system was an infant mouse model because the virus does not cause clinical signs of disease in other commonly used laboratory animals. Unfortunately, there is the possibility that our findings with ribavirin may not be directly applicable to primates because the metabolism of ribavirin in rodents is different from that in primates (Catlin et al., 1980; Ferrara et al., 1981).

The model, itself provided interesting observations on virus tropism. There was remarkable specificity in CCHF virus tissue tropism in infant mice. In some broad respects, the demonstrated pathogenesis was similar to some known aspects in humans. The relatively early association of CCHF virus with both Kupffer cells and hepatocytes found in this model bears relevance to human disease. A series of published autopsy findings was summarized by Hoogstraal (Hoogstraal, 1979). After extensive pathologic examination of many cases of fatal CCHF infection over a 4-year period, Karmysheva concluded that CCHF virus multiplied in cells (especially Kupffer cells) of the reticulo-endothelial system, in which specific antigens were detected by immunofluorescence. Gear described CCHF as a liver disease (Gear, 1988). Swanepoel et al. (1989) found a prolonged viremia (maximum titer found  $3.7 \log_{10}$  FFU/ml) and necrotic lesions in five of seven liver specimens taken from deceased patients. They also found that patients who died developed terminal multiple organ failure, including cerebral and liver among others. In this model, virus growth in brain and other organs was a terminal event despite a rather prolonged viremia.

Some murine coronaviruses and some arboviruses have a liver tropism in infant mice similar to that shown in these experiments (Ruebner and Miyai, 1962; Shope and Causey, 1962). Because of that, we have gone to elaborate lengths to eliminate intercurrent infection or contamination of reagents as an explanation for the experimental results. Developing and using a CCHF virus neutralizing monoclonal antibody to confirm identity of virus was one step in establishing its purity and identity.

Ribavirin treatment, in this murine model, was effective in (1) increasing the geometric mean time to death of treated animals, and (2) reducing the frequency of clinical signs and mortality. Demonstration of ribavirin efficacy *in vivo* was not totally unexpected because ribavirin has been shown to have *in vitro* activity against CCHF and many other Bunyaviruses (reviewed in Huggins, 1989; Monath, 1990). Nevertheless, *in vitro* and *in vivo* efficacy do not always correlate as in the case of yellow fever virus where *in vitro* effect in Vero cells is not duplicated by *in vivo* protection of primates (Huggins, 1989). Although drug efficacy was established, a suppressed liver cell infection and viremia continued for a relatively long period in those apparently healthy ribavirin-treated animals examined. In most cases, the liver infection was

apparently self-limiting because there was no subsequent mortality in animals held for observation. A prolonged depressed viremia during ribavirin treatment also occurs in Lassa fever virus-infected animals that ultimately survive (Jahrling et al., 1980; McCormick et al., 1986). The source of the viremia has not been described, but it may also be the liver based upon the disease signs and symptoms.

The source of the prolonged suppressed viremia in our experiments was apparently the liver. CCHF virus isolated from the liver of ribavirin-treated animals was more exclusively hepatotropic during limited passage in placebo-treated and ribavirin-treated mice than the parent virus. It did not infect organs other than the liver (and serum) *in vivo* and it did not grow well in CER or SW13 cells *in vitro*. The brain was relatively spared after *i.p.* inoculation; the above putative subpopulation appeared to be less neurovirulent for infant mice than did the parent virus. This is not an unusual result because drug-selected mutants have, in other studies, sometimes been less virulent than the parent virus (Field and Coen, 1986).

The present data suggest that virus was propagated in the liver of CCHF virus-infected, ribavirin-treated mice. A dose regimen (single dose, 50 mg/kg) that, with the parent virus, reduced virus titers and mortality was ineffective using virus harvested from the liver of ribavirin-treated animals. Nevertheless, an increased dose of drug (single dose, 100 mg/kg) did, indeed, substantially reduce virus titers in both the liver and serum even though mortality was not reduced. Repeated passage of the liver virus in ribavirin-treated mice (multiple dose, 100 mg/kg) resulted in a loss of infectivity. These latter two observations suggest that, while partial resistance was apparent, it may not be real. What is seen as partial resistance in this model may be related to a pharmacologic property of ribavirin unique to infant mouse tissue. For example, infant mouse liver cells could become resistant to the effects of low doses of the drug thereby making sensitive virus appear resistant as postulated elsewhere (Herrmann and Herrmann, 1977).

Moreover, resistance to ribavirin has never been described unlike the case with other antivirals such as acyclovir, idoxuridine, and BVDU (*E*-5(2-bromovinyl)-2'-deoxyuridine). The prevailing concept is that resistance to ribavirin is unlikely to develop because the drug shows multiple sites of antiviral action (Hall et al., 1983; Wilson et al., 1984; reviewed in Gilbert and Knight, 1986; Huggins, 1989). The theory is that resistance is more likely to happen with more narrow-spectrum viral inhibitors that act by inhibition of a single viral enzyme (Hall et al., 1983; Wilson et al., 1984; reviewed in Gilbert and Knight, 1986; Huggins, 1989). The reported inability to isolate resistant mutants has stimulated the question of technically classifying it as an antiviral agent (Herrmann and Herrmann, 1977). Factors other than direct antiviral effects are postulated to account for the apparent antiviral efficacy of this compound. Yet, available evidence suggests that it has both host cell and specific antiviral effects.

Most of the detailed studies on the antiviral effect of ribavirin have been

done with influenza viruses (reviewed in Gilbert and Knight, 1986). It has been well established that ribavirin changes cellular purine nucleotide metabolism through an inhibition of inosine monophosphate dehydrogenase, a key enzyme in the synthesis of guanosine nucleotides. However, there is also evidence of ribavirin-induced inhibition of influenza virus RNA-polymerase on the one hand (Eriksson et al., 1977), and the mRNA-capping enzyme, guanyl-RNA-transferase (Goswami et al., 1979) on the other. It has also been suggested that ribavirin inhibits influenza virus VRNA-polymerase through a more specific mechanism than decreasing cellular GTP pools (Wray et al., 1985). Nevertheless, efforts to develop ribavirin resistant mutants of influenza virus have been unsuccessful (Field, 1988).

Our methodology in using the animal model was similar to that used in defining poxvirus resistance to thiosemicarbazone (Appleyard and Way, 1966) amantadine-resistant influenza (Oxford et al., 1970) and acyclovir-resistant mutants of herpes simplex virus (Field, 1982). However, our use of similar methodology produced no conclusive evidence that CCHF virus resistance to ribavirin developed. Rather, a hypothesis more consistent with other ribavirin pathogenesis experiments is that ribavirin treatment of infected infant mice transiently selected for a CCHF virus subpopulation more highly hepatotropic than the parent virus.

It may be that ribavirin-induced selection of a virus subpopulation with an enhanced tropism for a particular target organ has occurred elsewhere. During ribavirin treatment of Machupo virus (arenavirus) infected rhesus monkeys, viremia was significantly reduced. In fact, by day 10, viremia was not detected. Nevertheless, all treated animals died. But, 80% of treated animals died with a late neurologic syndrome that was seen in only 20% of infected and placebo-treated control animals. It is presumed that the late neurological phase of the disease results from the inability of ribavirin to concentrate in the CNS (Stephen et al., 1980). While that is the primary concern, it is also possible that a virus subpopulation with greater tropism for the CNS initially survived ribavirin treatment at the dosage used and contributed to the disease process.

In the CCHF virus infant mouse model, the virus subpopulation isolated from ribavirin-treated mice was less neurotropic for infant mice than the parent population. Further study of this subpopulation with remarkably homogenous tropism may provide insight into virus-host cell interactions related to organ tropism. In the past, drug-selected virus variants have been useful in this type of research (Field and Coen, 1986).

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