Prophylactic ribavirin treatment of dengue type 1 infection in rhesus monkeys

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Summary

The prophylactic efficacy of the broad-spectrum antiviral nucleoside analog ribavirin against flavivirus infection in non-human primates was investigated in a blinded, placebo-controlled study of rhesus monkeys infected with dengue virus. Both placebo- and ribavirin-treated monkeys developed viremia, as measured by direct plaque assay on *Aedes albopictus* C6/36 cells. Peak viremia occurred between days 3 and 9 after infection. No significant differences in time of onset, duration, or level of viremia were observed between placebo- and ribavirin-treated monkeys. Ribavirin induced predictable and reversible anemia and thrombocytosis. Serum ribavirin reached maximum levels of 30 μM by day 4, which approximates the in vitro minimum inhibitory concentration for dengue virus. Ribavirin appeared ineffective as a prophylactic drug for dengue type 1 viral infection, as evaluated by the magnitude of viremia in this monkey model.

Ribavirin; Dengue; Rhesus monkey

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Introduction

Dengue viruses (types 1–4) are positive-strand RNA viruses in the single genus, flavivirus, of the family Flaviviridae (Westaway et al., 1985). These viruses are transmitted to humans by *Aedes* spp. mosquitoes and both the vectors and the disease are expanding to previously unaffected parts of the world (Gubler, 1988). The disease in humans is generally an acute, incapacitating, and self-limited febrile illness (Monath, 1986). Rarely in adults and more commonly in children, infections are associated with more serious clinical manifestations known as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Halstead, 1980).

Present therapy for dengue is limited to supportive care. Preventive measures have been aimed primarily at eliminating the vector mosquito populations but have proved only partially effective (Anon., 1980; Schliessman and Calheiros, 1974). In addition, there is no currently licensed dengue vaccine, although there are several encouraging early clinical trials with live-attenuated candidates (Bancroft et al., 1986).

Ribavirin is a nucleoside analog which has a broad-spectrum of in vitro antiviral activity, including significant efficacy against dengue (Ussery, 1981; Canonico, 1983). Ribavirin has also been shown to have significant in vivo activity against a number of other viruses (Stephen et al., 1980) and has proven effective in treating influenza (Gilbert et al., 1983), respiratory syncytial virus (Taber et al., 1983), Lassa fever (McCormick et al., 1986) and hemorrhagic fever with renal syndrome (Huggins et al., 1987). Clinical trials are underway using ribavirin in the treatment of Argentine hemorrhagic fever in Argentina (Enria et al., 1987). In addition, ribavirin has been shown to have some effectiveness against West Nile virus (a flavivirus) infection in suckling mice (Odelola, 1977). Similar studies done at this institute with ribavirin to treat neurovirulent yellow fever viral infection in rhesus monkeys showed that ribavirin does not alter the course of encephalitis, mortality, viremia, and liver dysfunction in that model (Peters et al., 1989). For dengue, there is no animal model of human disease, although chimpanzees, gibbons, and monkeys develop a measurable viremia after inoculation with certain strains of dengue virus (Scherer et al., 1978; Whitehead et al., 1970; Halstead et al., 1973). Therefore, in order to assess the potential for the use of ribavirin in dengue infections, we examined whether ribavirin would alter the course of viremia in rhesus monkeys infected with dengue virus.

Materials and Methods

Animals

Twenty healthy rhesus monkeys (*Macaca mulatta*) free of neutralizing antibodies to yellow fever and dengue type 1, 2, 3 and 4 viruses were selected for these investigations. The monkeys ranged in weight from 3 to 7 kg, were identified by chest tattoo, and were caged individually in squeeze cages. The monkeys were fed

twice daily with monkey chow supplemented with fresh fruit and were allowed water ad libitum. General animal care was in full accordance with the American Association for Accreditation of Laboratory Animal Care Guidelines for the care of nonhuman primates and appropriate standards of the United States Department of Agriculture; guidelines considered appropriate by the National Institutes of Health. Sedation with ketamine was used to minimize trauma associated with physical examination and phlebotomy. Animal rooms were lighted with fluorescent lights maintained on a 12-h diurnal cycle and were regulated to 70°F and 50% relative humidity (RH), avoiding extreme fluctuations.

Virus and viral assays

The challenge virus was dengue type 1, Western Pacific 74 strain, obtained as a lyophilized product supplied by Dr Kenneth Eckels, Walter Reed Army Institute of Research, Washington, D.C. The reconstituted virus sample contained 4×10^5 plaque forming units (PFU) per ml.

The infectivity of viral inoculum and serum samples was determined in established mosquito (*Aedes albopictus* C6/36) or mammalian (LLC/MK-2) cell lines. Mosquito cells were maintained at 28°C for propagation into confluent monolayers in Singh's mosquito culture medium (Gibco, Formula 78-0086 AJ) containing 10% fetal bovine serum and antibiotics. For virus assays, samples were diluted serially and inoculated onto confluent cell monolayers (6-well plates or 25 cm^2 flasks); overlaid with Hanks' balanced salt solution containing 2% lactalbumin hydrolysate, 5% fetal bovine serum, sodium bicarbonate, antibiotics, and 0.75% SeaKem agarose (FMC Bioproducts); and incubated at 35°C, 5% CO_2, with 90% RH for an additional 5 to 7 days (Hasty and Dalrymple, in preparation). Plaques were read after staining cells overnight with neutral red. Similar procedures were used for plaque assays in LLC/MK-2 cells.

Ribavirin

Ribavirin (Virazole) was obtained from Viratek, Inc. The drug was prepared for injection by resuspension and dilution in sterile water, USP, to a final concentration of 100 mg/ml. Sterile saline was the placebo. Injections were given through a 25-gauge 1-inch needle placed intramuscularly (IM) in the lower extremity. The dose of drug was chosen as the maximum dose that would be tolerated by the animals, considering previous studies that indicate significant toxicity at doses above 30 mg/kg/day (Canonico et al., 1984; Pifat, personal communication).

Radioimmunoassay, essentially as described previously (Austin et al., 1983), was used to quantitate levels of ribavirin in serum samples. In brief, 0.1 ml of sample containing ribavirin, 0.2 ml of radioimmunoassay buffer, 0.1 ml of diluted rabbit polyclonal anti-ribavirin serum prepared at USAMRIID, and 0.1 ml of ^{3}H-ribavirin (ICN Pharmaceuticals) were mixed in a polypropylene tube. After overnight incubation at 4°C, 0.07 ml of bovine serum globulin or horse serum and 0.5 ml of cold, saturated ammonium sulfate were added. Two hours later, the pellet was ob-

tained by centrifugation for 20 min at 1400 × g. The pellet was washed with 50% saturated ammonium sulfate and dissolved in 0.1 ml of deionized water. One tenth of an ml of 4 N HCl was added to all dissolved pellets as a quenching agent. Aquasol (10 ml) was added and the samples were counted in a liquid scintillation counter. Ribavirin concentrations in serum samples were determined from a standard curve with the lower limit of detection equal to 1 μM.

Clinical laboratory assays

Complete blood counts (including differential white blood cell and platelet counts), serum electrolytes, and liver function tests were performed on days −1, 6, 14, and 28 in the College of American Pathology-approved Clinical Laboratory, USAMRIID.

Serum samples for routine chemistry results were analyzed in an Abbott Spectrum Chemistry Analyzer (Abbott Laboratories, Irving, TX) with reagents, rinsing solutions, and standards manufactured by Abbott Laboratories.

Routine hematologic determinations were performed with the Ortho ELT-15 Hematology Analyzer, Ortho Diagnostics, Inc. using Ortho Diagnostics reagents and flushing solution. Calibration controls for white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hgb), and hematocrit (Hct) were from R and D Systems, Inc, Minneapolis, MN. Platelet calibration controls were from Streck Laboratories, Omaha, NB.

Study design

Monkeys were randomized by sex and weight into two groups of 10 each. Investigators handling the monkeys were given vials coded for each monkey. The content of the vial (i.e. ribavirin or placebo) was not disclosed to the investigators until all data, including viremia results, were obtained. All monkeys were given initial ribavirin or placebo injections on day −1. Injections were IM with a loading dose of 50 mg/kg followed by 10 mg/kg at 8-h intervals for 10 days. On day 0 (24-h after the first drug dose), all monkeys were inoculated IM with 2 × 10⁵ PFU of dengue type 1, Western Pacific 74 strain. The animals were checked twice daily for clinical signs of illness. Once daily from day −1 to day 10 then every 2 days until day 14, monkeys were sedated with ketamine (10 mg/kg; IM) for weighing, examination, and phlebotomy.

Results

Signs and symptoms of dengue

During the course of the study, all animals remained healthy and active. Two monkeys in the ribavirin group developed transient diarrhea on days 2 and 3 and one monkey in the placebo group exhibited occasional nosebleeds, evident on exam
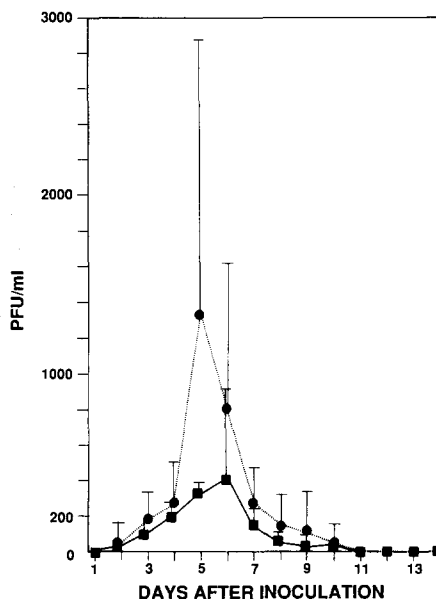
Fig. 1. Dengue type 1 viremia in rhesus monkeys treated with ribavirin. Serum samples taken daily on monkeys receiving ribavirin (......) or placebo (—) were assayed in duplicate for PFU on *A. albopictus* C6/36 cells. Data points are the average for ten monkeys per group. Error bars indicate two standard deviations from mean for each point.

on days 8 through 11. Regional adenopathy and rectal temperature greater than 39.5°C were observed in 8 of 10 placebo-treated and 7 of 10 ribavirin-treated animals. Average daily rectal temperatures were not statistically different between the ribavirin-treated and placebo control groups (data not shown).
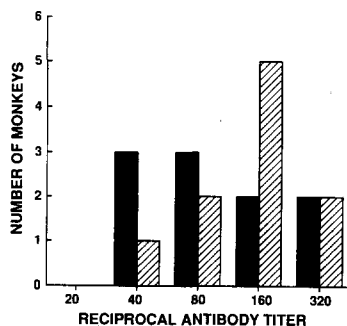

Fig. 2. Distribution of dengue type 1 neutralizing antibody response in monkeys 28 days after challenge. Serum samples taken on day 28 from each monkey were assayed individually to determine the 80% plaque-reduction neutralization antibody titer against dengue type 1 virus in LLC-MK2 cells. Solid bars, control; hatched bars, ribavirin.

Viremia

All monkeys developed viremia as measured by quantitative direct plaque assay of serum samples on *Aedes albopictus* C6/36 cells. Viral titers peaked on day 5 in ribavirin-treated animals and day 6 in controls (Fig. 1). The peak viral titer (PFU/ml serum) ranged from 229 to 4410 in the ribavirin group and 141 in 1587 in control monkeys, with averages of 1602 and 510, respectively. These differences were statistically indistinguishable. Direct plaque assays on LLC-MK-2 cells were uniformly less sensitive than assays on C6/36 cells (data not shown). Neutralizing antibody to challenge virus was present in all monkeys on day 28 (Fig. 2). Reciprocal titers resulting in an 80% reduction in plaques ranged from 40 to 320 for both
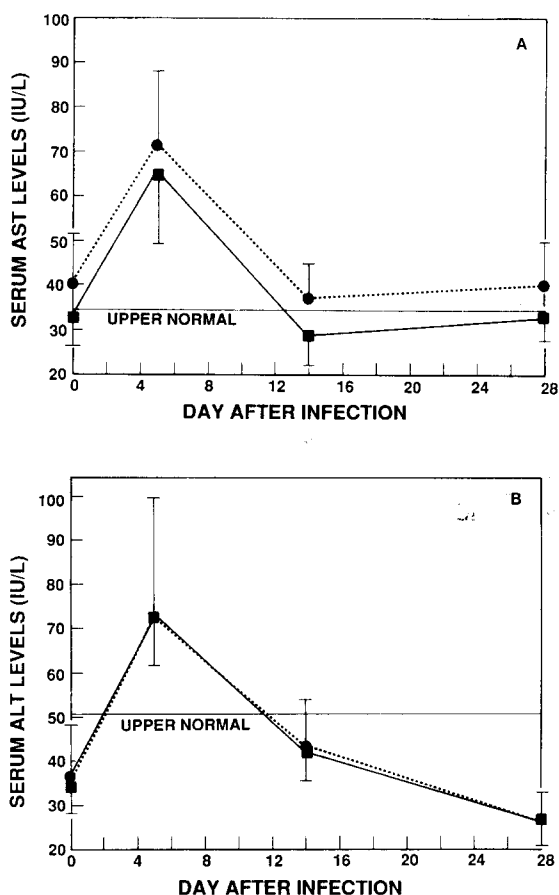

Fig. 3. Serum liver enzyme levels in rhesus monkeys infected with dengue type 1 virus. Serum samples obtained from each group at the indicated times were analyzed for aspartate aminotransferase (AST) (A) and alanine aminotransferase (ALT) (B) levels. Solid line indicates upper limit of normal. Error bars indicate two standard deviations from mean of 10 animals for ribavirin (.....) and placebo control (——).

groups. Geometric mean titers were 139 and 98 for ribavirin and placebo recipients, respectively.

Laboratory studies

Serum liver function tests were elevated to the same degree in both ribavirin- and placebo-treated groups on day 4 and returned to normal by day 14 (Fig. 3). These elevations were more than twofold above normal (Anderson, 1966).

Examination of hematologic parameters showed that the ribavirin-treated monkeys had a predictable and reversible decrease in hematocrit and increase in platelets (Fig. 4). These observations are consistent with the interpretation that the ribavirin dose was near the maximum tolerable dose that could be administered safely
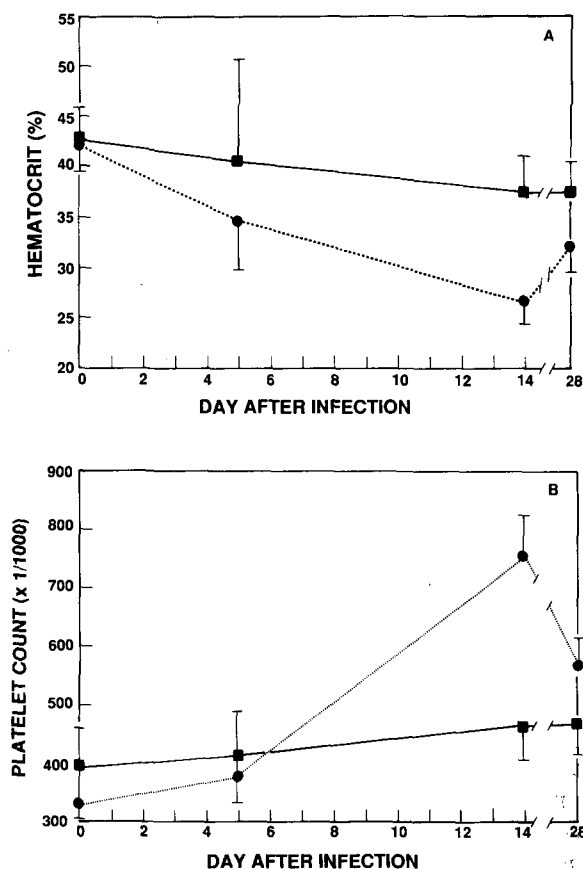

Fig. 4. Hematologic abnormalities in rhesus monkeys infected with dengue type 1 virus and treated with ribavirin. Blood samples obtained from each group of animals at the indicated times were analyzed for hematocrit (A) and platelet count (B). Error bars indicate two standard deviations from mean of 10 animals for ribavirin (.....) and placebo control (—) groups.
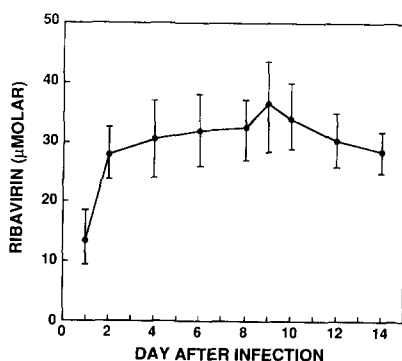
Fig. 5. Ribavirin levels in rhesus monkeys infected with dengue type 1 virus and treated with ribavirin. Serum samples obtained from ribavirin-treated animals at the indicated times were analyzed for ribavirin content. Data points indicate mean of 10 animals and error bars indicate two standard deviations from mean.

to these animals. Dengue infection alone had no effect on these parameters. No significant decrease in hematocrit occurred in control animals.

Ribavirin levels

Serum ribavirin levels were determined by radioimmunoassay on samples obtained immediately prior to the morning drug dose. Ribavirin reached maximum levels of approximately 30 μM (± 5 μM) (7.3 μg/ml) by day 4 and remained in that range through day 14 (Fig. 5).

Discussion

The rhesus model used in this study seemed an appropriate model of dengue 1 viral infection since all animals exhibited viremia and developed neutralizing antibody. It was of interest that over 75% of animals demonstrated mild symptoms of acute febrile infection, including lymphadenopathy, increased rectal temperatures, and transient elevations in serum transaminase levels; all consistent with dengue infection. Although liver enzyme levels have not been evaluated in other published studies, no significant changes were noted in prothrombin time or total protein; parameters that reflect liver function (Halstead et al., 1973). However, transient leukopenia with lymphocytosis can follow dengue infection in rhesus monkeys (Halstead et al., 1973) and lymphadenopathy was reported in half the chimpanzees inoculated with dengue viruses (Scherer et al., 1978). Our observations of increased liver enzyme levels were somewhat unexpected and warrant further evaluation since uninfected controls were not used. Furthermore, the increases in transaminases may have resulted from other causes, such as increased activity associated with daily handling and observation or direct muscle tissue

damage (releasing muscle enzymes that cannot be distinguished from liver enzymes) associated with three times daily IM injections.

One possible explanation for the ineffectiveness of ribavirin in preventing dengue infection in this study is that ribavirin did not reach levels necessary to inhibit viral replication. Studies in vitro indicate that the minimum inhibitory concentration (MIC) for dengue 1 is approximately 30 μM (Ussery, 1981). However, as measured by the hematologic side effects of anemia and thrombocytosis, ribavirin was administered at a maximum tolerated dose and was given prophylactically in order to obtain significant serum levels of the drug prior to dengue viremia. Furthermore, the serum concentration of ribavirin was at or above the in vitro MIC within 4 days of the start of therapy. Yet, viral replication was not affected.

Higher doses of ribavirin would be expected to produce higher serum levels of ribavirin. However, such doses would not be useful for several reasons. First, dramatic toxicity for rhesus monkeys has been noted at ribavirin doses higher than 30 mg/kg/day, to include anemia, thrombocytosis, and weight loss (Canonico et al. 1984; Pifat, personal communication). Secondly, the changes in hematocrit and platelet counts in our experiments were significantly higher than expected for a dose of 30 mg/kg/day based on previously published data (Cosgriff et al., 1984) and approached the changes observed at 100 mg/kg/day. Lastly, doses of ribavirin above 15 mg/kg for the treatment of human infections have been limited because of significant anemia and thrombocytosis. Although higher doses have been justified in diseases, such as Lassa fever, in which significant mortality is expected (Centers for Disease Control, 1988), such mortality is not seen with dengue fever and, therefore, it would not be clinically prudent to require ribavirin doses greater than 30 mg/kg/day.

The pharmacokinetics of ribavirin in man are best explained by a three-compartment model in which ribavirin enters the circulation, is rapidly incorporated into intracellular pools (in phosphorylated forms), and is then slowly released from intracellular pools after dephosphorylation to be excreted in urine and bile as various metabolites (Roberts et al., 1987). Thus, the value of measuring the serum levels of unphosphorylated ribavirin in order to predict therapeutic efficacy remains a question and assays for the phosphorylated forms of ribavirin would be useful.

Another possible explanation of the ineffectiveness of ribavirin to inhibit dengue virus replication in vivo is that ribavirin may not accumulate to similar levels in all non-neural tissues. Thus, it is conceivable that the tissues in which dengue virus replicates best (e.g. monocyte/macrophage cells) may not accumulate a sufficient amount of ribavirin to inhibit viral replication. This would also help to explain the in vitro observation that ribavirin suppresses the growth of dengue virus in LLC-MK2 cells but has no effect on dengue viral replication in human peripheral blood leukocytes (Koff et al., 1982). In addition, as there are documented differences between tissues with respect to the extent of de novo synthesis and salvage use of purines for metabolism (MacKinnon and Deller, 1973; Berman et al., 1980), such tissues may differ in their ability to concentrate the important antiviral forms of ribavirin.

The hematologic side effects of ribavirin limited the dose of ribavirin used in this study. However, if these side effects can be inhibited or reversed or derivatives of ribavirin that do not adversely affect hematopoietic cells can be found, there remains a possibility that higher serum levels of ribavirin could be reached and that such levels would possibly inhibit dengue viral replication. One encouraging report along these lines is the demonstration that recombinant human erythropoietin can limit the anemia associated with ribavirin (Pifat et al., 1990).

Ribavirin clearly exhibits a broad spectrum of antiviral activity and has been shown to be clinically useful in treating several viral infections associated with hemorrhagic complications (McCormick et al., 1986; Huggins et al., 1987). Thus, it is an attractive drug for the potential treatment of flavivirus infections, such as dengue, which may have hemorrhagic complications. However, this is the second study to demonstrate that ribavirin is ineffective in treating flavivirus infection in non-human primates (Peters et al., 1989). Ribavirin did not appear to alter the onset, degree, or duration of viremia in monkeys infected with dengue virus. Furthermore, there were no significant differences in fever, regional adenopathy, or liver function enzyme elevations between the ribavirin- and placebo-treated animals. Although it has been suggested that an antiviral drug capable of limiting viremia might improve survival and, perhaps, speed recovery, ribavirin clearly failed to reduce dengue viremia in this study; based on these results, ribavirin would appear of little potential benefit for treating dengue infections in humans.

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