Aerosol and Intraperitoneal Administration of Ribavirin and Ribavirin Triacetate: Pharmacokinetics and Protection of Mice against Intracerebral Infection with Influenza A/WSN Virus

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Ribavirin is active *in vitro* but not *in vivo* against a number of viruses capable of causing encephalitis. Ribavirin triacetate (RTA), a lipophilic derivative, has been reported to be more effective than ribavirin in protecting animals from encephalitis. By using an influenza A/WSN virus encephalitis model, we demonstrated that RTA administered by small-particle aerosol was able to decrease the death rate and increase the time of survival. To determine if this beneficial effect was due to increased delivery of drug, the pharmacokinetic properties of ribavirin and RTA when administered as an aerosol or by intraperitoneal injection were examined. Aerosol administration of ribavirin or RTA gave significantly higher concentrations of ribavirin in the lungs and serum of mice than did intraperitoneal injection. There was no difference, however, in ribavirin levels when either ribavirin or RTA was administered by small-particle aerosol. In brain tissue, ribavirin concentrations increased with time and did not appear to decrease as rapidly as in lungs and serum. Mean peak ribavirin concentrations in the brain were higher following aerosol administration of ribavirin than RTA, and both were higher than that following intraperitoneal injection of either drug. Administration of ribavirin or RTA by intraperitoneal injection failed to protect mice from a lethal intracerebral inoculation of influenza A/WSN virus, while aerosolized RTA did protect mice. The pharmacokinetics of ribavirin in brain tissue following aerosol administration of either drug did not explain the advantage of RTA over ribavirin in protecting mice from intracerebral infection with influenza A/WSN virus.

Ribavirin is a broad-spectrum antiviral agent with *in vitro* activity against a number of viruses capable of causing encephalitis. Although ribavirin has been shown to cross the blood-brain barrier in both mice (4) and humans (1, 12), it has not been very effective in preventing neurological symptoms or diseases caused by viruses. In a herpes simplex virus type 1-mouse encephalitis model, ribavirin administered either subcutaneously or intraperitoneally (*i.p.*) did not appreciably alter the course of herpes simplex virus-induced encephalitis, although direct injection of the drug into brains 3 h prior to virus inoculation resulted in a significant increase in survivors (14). In a Punta Toro virus-mouse model, no effect was observed when ribavirin was administered subcutaneously to mice infected intracerebrally with the Punta Toro virus strain Balliet, even though treatment was begun 36 h before viral inoculation (16). On the other hand, suckling mice treated with 50 mg of ribavirin per kg of body weight per day begun on day 10 after infection with Hantaan virus had a delayed, but greater than 10-fold reduction of virus in the brains of treated animals (5).

In rhesus monkeys, ribavirin treatment of dengue virus type 1 infection (10), neurovirulent yellow fever (13), and experimental Argentine hemorrhagic fever (11) infections has not been effective. In humans, oral ribavirin therapy for subacute sclerosing panencephalitis failed to show clear improvement in the neurological status of either of two patients (12). These data suggest that ribavirin is not effective in treating viral encephalitis when administered by conventional routes.

Derivatives of ribavirin have also been tested in several animal encephalitis models. Ribamidine, the 3-carboxamide derivative, administered subcutaneously to mice infected intracerebrally with the Balliet strain of Punta Toro virus had a moderate inhibitory effect on virus titers in the brains of infected, treated mice but did not increase the number of survivors or mean survival time (15). Two lipophilic derivatives have also been tested. Subcutaneous administration of tributylribavirin increased the mean time to death but did not enhance survival time in guinea pigs infected intracerebrally with Argentine hemorrhagic fever virus (6), while *i.p.* infection of ribavirin triacetate increased the mean survival time and decreased the rate of death in mice infected intracerebrally with dengue virus type 2 (9). These data suggest that derivatives of ribavirin may be more effective than ribavirin. A proposed mechanism is that they are metabolized or cleared more slowly, thus giving them a "depot effect" in lipid-containing tissues.

We have previously shown that ribavirin administered by small-particle aerosol (SPA) reaches the brain and achieves concentrations which should be sufficient for antiviral effectiveness (4, 8). However, parenteral administration of ribavirin was not compared. We hypothesized that SPA administration of ribavirin might achieve higher concentrations in the brain because a drug delivered by SPA would preferentially go from the lungs to the brain before first passing through the liver and kidneys; increased levels in the brain would clearly be more advantageous for the treatment of encephalitis. In this report, we describe the pharmacokinetics and effects of ribavirin and ribavirin-2',3',5'-triacetate

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(RTA) when administered as SPAs or injected i.p. on our neurotropic influenza A/WSN virus encephalitis model (19).

MATERIALS AND METHODS

Materials. Ribavirin and RTA were provided by Viratek (Costa Mesa, Calif.). HPLC-grade water, methanol, and acetonitrile were obtained from J. T. Baker (Phillipsburg, N.J.). HPLC-grade ammonium phosphate (monobasic) and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, Pa.). Ammonium acetate, ammonium hydroxide, and formic acid were of the highest grade obtainable; all solutions containing these chemicals were made in HPLC-grade water. Ultrafiltration of biological samples was performed with Amicon CF25 ultrafiltration cones (Amicon, Danvers, Mass.) soaked in HPLC-grade water for 1 h prior to use.

Mice. Six- to eight-week-old (25 to 28 g), random-bred CD-1 mice, obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), were used in all experiments. The animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum. Mice were lightly etherized and inoculated intracerebrally with 15 to 20 PFU of influenza A/WSN virus in 5 μ l (day 0). The 50% lethal dose for this system is <10 PFU (19). Mice were observed for mortality for 21 days.

Determination of median 50% effective dose (ED₅₀). Antiviral assays were performed in 96-well tissue culture plates. All dilutions and tissue culture suspensions were prepared in minimal essential medium containing antibiotics and 2% fetal calf serum (2% FCS-MEM). Ribavirin and RTA (0.05 ml) were added in quadruplicate to the first wells of the test plates containing a subconfluent monolayer of MDCK cells (ca. 3×10^3 cells) and were diluted by using serial twofold dilutions. Approximately 100 median (50%) tissue culture infectious doses of influenza A/WSN (H1N1) virus in 0.05 ml were added. All plates were incubated at 37°C in a 5% CO₂ incubator for 3 to 5 days. The ED₅₀ was calculated by determining the concentration of antiviral drug completely inhibiting the cytopathic effect in 50% of the wells compared with virus control wells.

SPA treatment. Mice were placed in sealed plastic cages and exposed to aerosols as described previously (4, 21). The aerosol generator and particle characteristics were the same as previously described (7, 20). Drug concentrations were based on the assumption that the active component of RTA was ribavirin (see Results, *in vitro* ED₅₀). The average concentration of ribavirin or RTA delivered in the high-dose aerosol was 600 μ g of ribavirin or ribavirin equivalent of RTA per liter of air for reservoirs containing 60 mg of ribavirin or 90 mg of RTA per ml, respectively. The treatment period for infected mice was for 2 h, twice daily (7 am and 3 pm) for 10 days (days +1 through +10). The estimated dose of drug retained by the mice was calculated from the amount of drug in the aerosol, the minute volume, and duration of treatment as previously described (4, 21). In the pharmacokinetic experiments, three randomly chosen animals were used for each datum point. Blood (ca. 0.25 ml) was removed from the orbital sinus plexus of each animal; the animals were then sacrificed. Brains, consisting mostly of cerebral hemispheres, were removed, followed by the lungs. Blood was mixed with 0.25 ml of HPLC-grade water. Lungs and brains were washed free of adhering blood and homogenized in 1 ml of HPLC-grade water. All samples were stored at -70°C until processed.

i.p. administration. Mice (28 g) were inoculated i.p. twice

daily with 0.1 ml of ribavirin (7.1 mg/ml) or RTA (10.8 mg/ml), for a total daily dose of 50 mg/kg.

Quantification of ribavirin in biological tissues. Lung and brain tissues were suspended in 1 ml of HPLC-grade water and were homogenized with a glass bead beater. Blood was mixed with an equal volume (ca. 0.25 ml) of HPLC-grade water to lyse erythrocytes. All samples of lungs, blood, and brains were processed by centrifugation at $13,000 \times g$ for 5 min to remove debris, deproteinized by ultrafiltration through CF25 cones, and chromatographed on a phenylboronate affinity column as previously described (4, 17, 20). Ribavirin was quantified by high-performance liquid chromatography (HPLC) with monitoring at 207 nm. All measurements were made at ambient temperature on a Microsorb C18 stainless steel HPLC column (particle size, 5 μ m; length, 25 cm; inner diameter, 4.6 mm; Rainin Instrument Co., Emeryville, Calif.). A ribavirin (10 μ g/ml) standard was periodically processed (4, 17). It was assumed that RTA was deacylated *in vivo* to ribavirin and that ribavirin or its metabolic product(s) was the active antiviral agent (see Results). Thus, following ribavirin or RTA administration, tissues were assayed for ribavirin content. RTA (24 μ g/ml) could not be detected by the method used to quantitate ribavirin. Estimation of the ribavirin concentration in lungs, serum, and brain tissue was as previously described (4). An assay method for measuring RTA was developed involving perchloric acid (2.5% final concentration) precipitation followed by C18 reverse-phase HPLC with a mobile phase of 30% methanol and 70% water and detection at 207 nm. Although recovery was low (10 to 30%), RTA could be detected in spiked lung and blood samples at approximately 1 μ g/ml and in brain at 10 μ g/ml. Following aerosol or i.p. administration of RTA, we were unable to detect RTA in any of the tissues, possibly because of rapid deacylation of the RTA by the tissue homogenates or by blood.

Statistical analysis. Data analysis was performed with the True Epistat statistical package from Epistat Services, Richardson, Tex. *P* values are based on analysis of these data by the Student's *t* test with analysis of variance or by life table analysis.

RESULTS

***In vitro* ED₅₀.** The ED₅₀ of influenza A/WSN virus grown in MDCK cells was 94 μ M for ribavirin (23 μ g/ml) and >4 mM for RTA (>1,000 μ g/ml). Thus, RTA was inactive as an anti-influenza virus drug and must be converted to ribavirin by deacylation or to some other metabolite *in vivo*.

Intracerebral infection with influenza A/WSN virus. Ribavirin and RTA administered by daily aerosol or i.p. injection on days +1 through +10 after virus inoculation were used to treat mice infected intracerebrally with 15 to 20 PFU of influenza A/WSN virus. Equivalent ribavirin concentrations were given in each experimental condition. The estimated daily dose given by aerosol or by i.p. injection was 43 and 51 mg of ribavirin per kg, respectively. RTA aerosol protected mice from a lethal, neurotropic infection and increased survival time from 7.4 to 9.5 days (*P* = 0.031, life table analysis) (Fig. 1A), while in these experiments ribavirin did not. In two of five previous experiments, ribavirin aerosol (ca. 40 mg/kg/day) was protective (data not shown). i.p. administration of ribavirin or RTA failed to protect mice (Fig. 1B), while on two of three occasions, i.p. administration of RTA (50 mg/kg/day) increased survival time (*P* $\leq$ 0.05, Wilcoxon rank sum, two-tailed) but did not increase the number of survivors (data not shown).

FIG. 1. Effects of aerosol administration or i.p. injection of ribavirin or RTA on mortality in mice inoculated intracerebrally with 15 to 20 PFU of influenza A/WSN virus. As described in Materials and Methods, 15 mice were not treated (●) or were administered ribavirin (■) or RTA (▲) by SPA (A) or by i.p. injection (B) on days +1 through +10.

Pharmacokinetics following ribavirin and RTA aerosol administration. Protection experiments suggested that RTA administered by aerosol was an effective mode of treatment for influenza virus encephalitis. To determine if this protection was due to higher concentrations of ribavirin in the brains of these mice, pharmacokinetic experiments compared tissue levels of ribavirin in lungs, serum, and brain following aerosol or i.p. administration of either drug.

Following a single, 2-h aerosol treatment, ribavirin concentrations in lungs of mice given ribavirin were twofold higher than in animals given RTA (Fig. 2A). In serum and brain, ribavirin concentrations were similar in animals given either ribavirin or RTA (Fig. 2B and C). Patterns of deposition and clearance of ribavirin and RTA in lungs were similar to each other and to that previously reported for ribavirin (4). At 24 h posttreatment, there was no difference in the pattern of clearance or the concentration of ribavirin when either ribavirin or RTA was administered. This suggested that RTA was not acting as a pool for the sustained release of ribavirin.

When ribavirin concentrations were monitored during a 10-day SPA treatment period, we observed that when either ribavirin or RTA was administered, peak ribavirin levels in lungs decreased continuously from days 1 to 10 ($P < 0.02$) (Fig. 3A). The concentration of ribavirin in lungs just prior to the start of each treatment with either drug (i.e., trough levels) remained low and constant. Peak ribavirin concentrations in serum reflected lung levels but were generally lower, while trough levels increased over the 10-day ribavirin or RTA treatment period ($P < 0.05$) (Fig. 3B). In brain tissue, both peak and trough ribavirin concentrations increased by day 5 following ribavirin administration ($P = 0.084$ and $P < 0.001$, respectively) (Fig. 3C). Trough ribavi-

FIG. 2. Pharmacokinetics of ribavirin in lung (A), serum (B), and brain (C) following a single continuous administration of SPA for 2 h with 60 or 90 mg of ribavirin (■) or RTA (●) per ml, respectively, in the reservoir. Three mice were used for each time point. Tissues were obtained and processed as described in Materials and Methods. Ribavirin levels were measured by HPLC. Values represent the means found for three mice.

rin concentrations also increased by day 10 following RTA administration ($P = 0.009$). Thus, ribavirin appeared to accumulate in the brain over the 10-day treatment period and to reach a concentration of about 3 to 6 nmol per brain or an estimated 75 to 150 μM (4), a concentration greater than or equivalent to the ED_{50} value determined for this virus.

Pharmacokinetics of i.p. administration of ribavirin and RTA. The concentration of ribavirin in lungs following i.p. administration of ribavirin or RTA was similar and was about 5 to 10% of the pulmonary concentration following aerosol administration (Fig. 4A). Peak levels were achieved by the first time point (30 min), remained at those levels for about 2 h, and were cleared similarly for both drugs. In serum, there was some suggestion that RTA at the earliest time points might reach higher concentrations than ribavirin

FIG. 3. Concentration of ribavirin in lung (A), serum (B), and brain (C) following twice daily administration of ribavirin and RTA by SPA. Methods are as described in the legend to Fig. 2. Double and left narrow crosshatched bars: peak and trough levels (respectively) of ribavirin following SPA administration of ribavirin (left half of graph). Right narrow and left wide crosshatched bars: peak and trough levels (respectively) of ribavirin following SPA administration of RTA (right half of graph). Assays were performed on days 1, 5, and 10.

(Fig. 4B); however, at 1 h postinoculation following multiple injections, there was no difference between the two compounds (Table 1). Following multiple injections, peak serum ribavirin concentrations were about 30% of the concentration following aerosol administration (Table 1; $P < 0.001$, Student's t test, two-tailed) but were similar following administration of either drug. Clearance of ribavirin from serum over 8 h was also similar, suggesting that RTA was rapidly being metabolized to ribavirin (Fig. 4B). Mean trough ribavirin levels were similar following administration of either drug. In brain tissue, RTA appeared to reach higher concentrations following the first i.p. injection (Fig. 4C).

FIG. 4. Pharmacokinetics of ribavirin in lung (A), serum (B), and brain (C) following two i.p. injections of ribavirin (■) or RTA (●). Methods are as described in the legend to Fig. 2. Mice were injected at 0 and 8 h with 0.1 ml of either drug.

However, after multiple injections of either drug, both peak and trough concentrations of ribavirin were similar (Table 1).

A summary of the peak and trough concentrations for ribavirin following multiple aerosol or i.p. administrations of ribavirin or RTA over a 10-day treatment period is presented in Table 1. In lungs, mean trough values remained low and did not indicate accumulation of drug over time whether either drug was administered by aerosol or by i.p. injection. Following aerosol administration of either compound, mean peak and trough drug concentrations in serum were similar; in contrast, following i.p. injection of RTA, mean peak ribavirin levels were four times higher than trough levels (Table 1; $P = 0.009$, Student's t test, two-tailed). In brain tissue, mean peak ribavirin concentrations following aerosolization were higher after ribavirin administration than RTA administration. For either drug, peak concentrations

TABLE 1. Ribavirin concentrations in tissues following ribavirin or RTA treatment^a

Level, route of administration, and drug	Concn in lungs (nmol/lung)	Concn in serum (μM)	Concn in brain (nmol/brain)
Peak^b			
Aerosol			
Ribavirin	60.0 ± 31.0 ^c	27.1 ± 11.2 ^c	4.95 ± 1.02 ^{c,d}
RTA	29.7 ± 16.2 ^c	20.7 ± 5.3 ^c	2.71 ± 0.87 ^{d,e}
Intraperitoneal			
Ribavirin	4.27 ± 2.61 ^c	8.0 ± 2.4 ^c	1.54 ± 0.73 ^c
RTA	2.30 ± 0.50 ^{c,f}	8.2 ± 3.6 ^{c,f}	1.94 ± 0.83 ^e
Trough^g			
Aerosol			
Ribavirin	12.8 ± 1.5 ^e	21.2 ± 11.8 ^e	4.46 ± 5.17
RTA	10.1 ± 4.7 ^e	25.1 ± 14.2 ^e	3.70 ± 2.00
Intraperitoneal			
Ribavirin	1.74 ± 2.04 ^e	4.61 ± 3.13 ^e	1.35 ± 1.54
RTA	0.79 ± 0.48 ^e	2.00 ± 1.11 ^{e,f}	0.90 ± 0.78

^a Values are mean concentrations of days 1, 5, and 10 and of days 1, 2, 3, and 5 twice daily aerosol and i.p. treatments, respectively.

^b Peak levels were determined at 2 and 1 h posttreatment for aerosol and i.p. administration, respectively.

^c $P < 0.003$, aerosol versus i.p. administration.

^d $P < 0.05$, ribavirin versus RTA concentrations.

^e $P \leq 0.03$, aerosol versus i.p. administration.

^f $P < 0.01$, peak versus trough concentrations.

^g Trough levels were determined at 8 h posttreatment for all groups.

following aerosol administration were greater than those following i.p. injection. Trough concentrations of ribavirin following administration of either drug by aerosol or i.p. injection were similar.

DISCUSSION

We have previously shown that when influenza A/WSN virus replicates in the brain, an encephalitis occurs, characterized by perivascular cuffing, numerous foci of lymphocytic infiltrates in the meninges, perivascular infiltrates in the brain, astrocytic reactions throughout the cortex, and foci of rarefaction in the white matter (19). This extensive pathology was caused by an inoculum of less than 10 PFU of intracerebrally inoculated virus and led to death in 4 to 8 days. In the present study, RTA administered on days +1 through +10 by SPA protected mice from a lethal intracerebral infection with the neurotropic influenza virus A/WSN. The data suggest that to treat this infection, drug must reach the brain quickly (i.e., aerosol is better than i.p. administration), at concentrations sufficient to inhibit influenza A/WSN virus replication (i.e., $\geq 100 \mu\text{M}$) (8), and for a longer period of time than that necessary for the treatment of pulmonary influenza (i.e., ≥ 10 versus 4 days) (20, 21). Since RTA (and less often ribavirin) protected mice when given by aerosol and not when given i.p., our hypothesis was that aerosol administration allowed the drug to get to the brain more directly via the lungs and at higher concentrations than did i.p. injection (i.e., reducing "first pass" effects of the liver, kidneys, etc.) and that the lipophilic nature of RTA might allow a more prolonged and sustained release of ribavirin. Since there is no evidence that RTA can be directly metabolized to a phosphate derivative or to another metabolite without initially being converted to ribavirin, we have assumed that ribavirin is the active drug that is converted to its phosphate derivatives.

The failure of i.p. administered ribavirin to protect mice

may be attributable to the statistically significantly lower concentrations of ribavirin seen in animals given the drug i.p. compared with the concentrations seen in animals given the drug in aerosol form in all three tissues assayed (Table 1). The concentrations of ribavirin in brain tissue reflected concentrations in serum, which in the case of aerosol administration also reflected the concentrations in lungs. Part of the reason for higher serum concentrations following aerosol administration may be oral ingestion of the drug deposited on the animal's fur by subsequent preening. Of the ingested drug, only 45% would be bioavailable (3). Since the mean concentration of ribavirin following RTA administration was not higher than that following ribavirin treatment, we conclude that RTA was rapidly converted to ribavirin *in vivo* and did not give rise to sustained ribavirin tissue concentrations, as might be expected if RTA was acting as a ribavirin depot and slowly being metabolized to ribavirin.

Ribavirin concentrations in brain tissue increased with prolonged aerosol administration of either ribavirin (Fig. 3) (3) or RTA. With time, mean peak and trough levels became equivalent (3 to 6 nmol per brain, 75 to 150 μM). Aerosol administration gave higher peak concentrations of ribavirin when either ribavirin or RTA was given compared with i.p. injection. However, although trough levels were higher following ribavirin or RTA aerosol administration, they were not statistically significantly different. While the concentrations of ribavirin in the brain following aerosol administration of either drug appeared to be sufficient to inhibit virus replication, the level was such that daily fluctuations in individual animals may have decreased the concentration to below the ED_{50} value, causing the variability seen in the RTA and ribavirin protection studies.

Calculations of the area under the curve (AUC) following a single aerosol or i.p. treatment (Fig. 2) were made. Ribavirin concentrations were higher in the lungs and serum following aerosol administration of ribavirin than following administration of RTA (ratio, 0.6); however, in brain tissue, ribavirin concentrations were slightly higher following aerosol administration of RTA (ratio, 1.26). The greater AUC for RTA was due to a single datum point (8 h), and over a 10-day treatment period, administration of aerosolized ribavirin gave higher ribavirin concentrations (Table 1 and Fig. 3). i.p. administration of RTA also gave higher brain concentrations of ribavirin (ratio, 1.59), although the total AUC was less than 10% of the aerosol AUCs. These data suggest that aerosol administration is as good as, or better than, i.p. injection for attaining a concentration of ribavirin in the brain sufficient to inhibit influenza A/WSN virus replication.

It has been estimated that concentrations of ribavirin in the cerebrospinal fluid of mice following ribavirin aerosol administration may reach 75 to 150 μM , a level sufficient to inhibit influenza A/WSN virus replication. It should be noted, however, that following SPA administration, the concentration of ribavirin in the cerebrospinal fluid of children (2) and adults (1, 12) has been reported to be in the range of 1 to 10 μM and similar to or less than the patient's serum ribavirin concentration. This discrepancy may be due to the metabolism of ribavirin in the brain and in cells lining the ventricles, causing a reduction in concentration at the site of the spinal tap. Since ribavirin is converted by cells to ribavirin-5'-triphosphate, the active metabolite against influenza virus (3), a more appropriate evaluation of the effectiveness of ribavirin and RTA treatment would be to determine ribavirin-5'-triphosphate concentrations in brain tissue.

While the pharmacokinetic analysis did not conclusively show why RTA and occasionally ribavirin administered by

aerosol was superior to i.p. injection for increasing survival time and rates, it did show that the administration of these aerosols was an effective way to deliver ribavirin not only to the lungs and the systemic circulation but also in a noninvasive way to the brain.

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