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Antiviral Research 67 (2005) 38–45
Antiviral Research
www.elsevier.com/locate/antiviral

Error-prone replication of West Nile virus caused by ribavirin

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Received 25 October 2004; accepted 5 April 2005

Abstract

Ribavirin has been reported to cause error-prone replication and viral extinction in RNA viruses. The antiviral activity of ribavirin against West Nile virus (WNV) was evaluated in various cell lines to select a model in which mutagenic effects could be studied. The antiviral activity was greatest in HeLa cells as compared to CV-1, L929, Vero, or MA-104 cells. WNV was also passaged sequentially in cell monolayers treated with ribavirin to determine whether cumulative mutations could lead to viral extinction in these cell lines. The virus was abrogated in HeLa cells after 4 passages, while high viral titers persisted after many passages in other cells. A molecular clone of WNV was propagated in HeLa cells treated with 15 μg/mL ribavirin, and sequencing of viral genome segments revealed significant increases in transition mutations, demonstrating that ribavirin induced error-prone replication. The relative infectivity of viral RNA synthesized in the presence of ribavirin was shown to be reduced compared with untreated controls. These data support the hypothesis that error catastrophe is one of the modes of action for ribavirin against WNV.

Keywords: West Nile virus; Error catastrophe; Ribavirin; Antiviral; Mutagenesis

1. Introduction

West Nile virus (WNV) was observed in North America for the first time in 1999 with the outbreak in New York. The virus is usually transmitted by mosquitoes from an avian host and affects humans, birds, horses, and other animals (Petersen and Roehrig, 2001). WNV spread quickly throughout the United States and has resulted in over 12,300 human cases and hundreds of human fatalities in the years 2003–2004 (CDC, 2004). This outbreak has led to heightened interest in WNV research and an increased need to develop antiviral therapies against the virus.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a broad-spectrum antiviral agent with several known mechanisms of action. Its most widely known mechanism is inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depression of cellular guanosine triphosphate (GTP) levels (Streeter et al., 1973; Leyssen et al., 2005). Other mechanisms include inhibition of 5′ mRNA capping (Goswami et al., 1979; Scheidel and Stollar, 1991), RNA chain termination in some viruses such as reovirus (Rankin et al., 1989), and direct inhibition of the viral RNA polymerases as in influenza virus (Eriksson et al., 1977) and vesicular stomatitis virus (VSV) (Fernandez-Larsson et al., 1989). Ribavirin can also decrease translation efficiency, as in VSV (Toltzis and Huang, 1986), and may modulate host immune responses as shown in human patients (Fang et al., 2001) and lymphocytes (Joksic et al., 2000). More recently, ribavirin has been identified as a selective viral mutagen (Crotty et al., 2000).

RNA viruses exist as "quasispecies," which are a mixture of related genomes that share a consensus sequence and a broad mutant spectrum (Ruiz-Jarabo et al., 2000). Therefore, RNA viruses function on the edge of mutation crisis, and an increase in the average error frequency can result in loss of

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doi:10.1016/j.antiviral.2005.04.002

genetic information and decreased viability; this is termed "error catastrophe" (Crotty et al., 2000; Sierra et al., 2000). Ribavirin can be incorporated by viral polymerase and can base-pair equivalently with both cytidine and uridine (Crotty et al., 2000). Ribavirin has been shown to cause error-prone replication in poliovirus (Crotty et al., 2001), GB virus (Lanford et al., 2001), Hantaan virus (Severson et al., 2003), and hepatitis C virus (Contreras et al., 2002). Mutations may accumulate as a virus goes through multiple rounds of replication, leading to inactivation of the viral population, as shown with 5-hydroxydeoxycytidine against human immunodeficiency virus (HIV) (Loeb et al., 1999), 5-fluorouracil against lymphocytic choriomeningitis virus (Grande-Perez et al., 2002), and 5-azacytidine and 5-fluorouracil against foot-and-mouth disease virus (Sierra et al., 2000). In these prior studies, the virus populations were extinguished within 13–21 sequential passages in the presence of the mutagen. We hypothesized that cumulative mutations of this sort would lead to error catastrophe of WNV.

The in vitro antiviral activity of ribavirin varies depending on the cell line used (Sidwell, 1980), which is likely due to phosphorylation differences (Smee et al., 2001) and differences in intracellular GTP depletion (Smee et al., 2001; Leyssen et al., 2005). Therefore, antiviral activity was assessed in multiple cell lines to identify one in which ribavirin had sustained antiviral activity against WNV for use in studying mutagenic effects. Mutations were then studied by direct sequencing of viral genome segments replicated in HeLa cells treated with ribavirin. Our hypothesis was that ribavirin would induce mutations that would lead to decreased infectivity of the nascent viral population.

2. Materials and methods

2.1. Viruses, cells, media, and drug

The wild-type virus used was isolated from crow brain in New York (designated CDC 996625) and propagated in MA-104 cells. A WNV clone was provided by Dr. Vladimir Yamshchikov of the University of Virginia, accession #M12294. The NY isolate is a lineage I strain, which is virulent in humans, and the viral clone is from lineage II, which is less virulent (Yamshchikov et al., 2004). Cells used were CV-1 African green monkey kidney fibroblasts, ATCC CCL-70; L929 BALB/c mouse fibrosarcoma cells, ATCC CCL-1; HeLa human cervical adenocarcinoma, ATCC CCL-2; MA-104 African green monkey kidney epithelial cells (BioWhitaker, Walkerville, MD); and Vero 76 African green monkey kidney epithelial cells, ATCC CCL-1587. Minimal essential medium (MEM) with 0.18% sodium bicarbonate and 10% fetal bovine serum (FBS) was used for cell growth and the same medium with 1% FBS was used for antiviral assays. Ribavirin was from ICN Pharmaceuticals, Costa Mesa, CA.

2.2. Virus titers

Infectious virus throughout the study was titrated by 50% cell culture infectious dose ($CCID_{50}$) assays as previously described (Smee et al., 1992). Briefly, each virus solution was diluted serially in 10-fold increments and added in quadruplicate to Vero 76 cell monolayers in 96-well, flat-bottomed microplates (Corning Constar®). Each well was scored visually for cytopathic effect (CPE) after 6–7 days of incubation at 37 °C with 5% CO_2. The virus titer was calculated by extrapolation and reported as the $\log_{10}$ $CCID_{50}$/0.1 mL.

2.3. Antiviral efficacy and toxicity of ribavirin

The antiviral efficacy of ribavirin was determined by seeding triplicate wells of 96-well, flat-bottomed with $2–5 \times 10^4$ cells per well then incubating for 1 day. The subconfluent monolayers were then treated with serial dilutions of the drug or control. CV-1, MA-104, Vero and L929 cells were immediately infected with 5 $CCID_{50}$ (multiplicity of infection $\approx 10^{-4}$) of WNV and HeLa cells with 150 $CCID_{50}$ of WNV (MOI ≈0.004). The minimal multiplicity of infection (MOI) was used for initial antiviral assays to provide the greatest sensitivity to antiviral effects. Plates were incubated at 37 °C with 5% CO_2 in MEM with 1% FBS. Separate plates were evaluated at 2 days and 6 days to determine if the antiviral effect would be sustained over time. Cells were frozen, thawed, and then homogenized by pipetting. Lysates of triplicate wells were pooled for virus titer assays as described above. The 90% effective concentration (EC_{90}) of the drug was calculated using linear regression to determine the dose that reduced the virus titer by one $\log_{10}$ compared to untreated controls. Tests were repeated in triplicate. Drug toxicity (50% inhibitory concentration, IC_{50}) was determined by visual CPE in each cell line and also by neutral red vital stain assays in HeLa cells, as described elsewhere (Smee et al., 1988; Morrey et al., 2002).

2.4. Viral extinction

Viral extinction was tested by serial passage in subconfluent monolayers treated with ribavirin. Cell monolayers ($4.0 \pm 0.6 \times 10^5$ cells per well) were grown 1 day in MEM containing 5% FBS and 0.18% sodium bicarbonate, and a prescribed concentration of ribavirin. Test wells of L929, MA-104 and CV-1 cells had 100 μg/mL ribavirin added. HeLa cells had 47 μg/mL ribavirin added because 100 μg/mL yielded no virus, and 32 μg/mL yielded sporadic results due to putative break-through mutants in some replicate wells. Ribavirin has been shown to concentrate 23-fold inside L929 cells within 13.5 h by facilitated diffusion (Rankin et al., 1989), so pretreatment was done to load the cells with ribavirin before viral challenge. Triplicate wells of 24-well flat-bottomed microplates were then inoculated with 5000 $CCID_{50}$ of WNV (MOI ≈0.01) in MEM with 2% FBS for CV-1, MA-104 and HeLa cells, and approximately 2.5×10^5

$CCID_{50}$ (MOI ≈0.6) for L929 cells. The multiplicity of infection was intended to allow multiple rounds of replication and infection in each passage that would presumably promote accumulation of ribavirin-induced mutations with each passage. Vero cells were not used in this extinction assay based on low activity of ribavirin in the prior antiviral assays. An equal volume of fresh MEM was added with or without drug to achieve the final drug concentration in MEM with 1% FBS and 25 μg/mL of gentamicin. After 3 days of incubation at 37 °C, plates were frozen at −80 °C for >2 h, thawed, and the lysates were mixed by pipetting. Lysates were then diluted 1/100 in MEM with 2% FBS and added to fresh cells, and an equal volume of MEM with or without drug was added as before. The 1/100 dilution was made to reduce the effects of defective interfering particles and aged media; it was based on preliminary studies that showed maximum virus yield in each passage with a 1/100 dilution. This process was repeated for 22 passages in L929 cells, 26 passages in CV-1 cells, 7 passages in MA-104 cells, and 5 passages in HeLa cells. All virus homogenates from HeLa cells and selected passage numbers from the other cell lines (Fig. 2) were assayed for virus yield. The virus extinction study was also repeated in HeLa cells with a minimal possible dilution of 1/4 between passages to show that the virus was not eliminated by dilution alone.

2.5. WNV infectious clone

WNV clonal DNA (Yamshchikov et al., 2001) (accession #M12294) was transfected into DH5-α-chemically competent cells (Invitrogen, Carlsbad, CA). DNA was purified with the Plasmid Maxi Kit™ (Qiagen, Valencia, CA). RNA was transcribed in vitro using SP6 polymerase (mMessage mMachine Kit™, Ambion, Austin, TX). RNA was then transfected into Vero 76 cells by electroporation in Dulbecco's phosphate buffered saline (DPBS) at 960 mf and 0.30 kV for 12.4 ms (adapted from GenePulser™ Electroprotocol 144) and the virus grown in MEM with 10% FBS to yield 6.3×10^6 $CCID_{50}$/mL. The viral clone was propagated on HeLa cells for 3–4 days, as in the virus extinction study above, with 15 μg/mL ribavirin alongside controls with no ribavirin. RNA was purified using Trizol™ and RNA Easy™ (Qiagen) methods.

2.6. RT-PCR and sequencing

RNA was reverse transcribed and PCR-amplified using the Reverse Transcriptase RNA PCR Kit (GeneAmp®, Applied Biosystems, Foster City, CA,) or StrataScript™ (Stratagene, La Jolla, CA) for cDNA and PfuTurbo® (Stratagene). Initial primers were 5′-tggatgacgacggaagacatg (positions 10093–10113) and 5′-gggtctcctctaacctctag (positions 10812–10831 complement) in the NS5 protein and untranslated region (UTR) of the genome (Pierre et al., 1994). A second primer set imbedded in the same regions was used to yield additional clones, 5′-atccatgtggcaatcaatca (positions 10273–10292) and 5′-gtggtctgacactgggcttt (positions 10591–10610 complement). A third primer set was designed in the E protein region of the genome, 5′-cgggaaacactgatggagtt (positions 1660–1679) and 5′-acggtcaccagtcttccaac (positions 2008–2027 complement). Approximately 55% of bases sequenced were from the 3′ untranslated region and 45% from coding regions (35% from the NS5 region and 10% from the E protein region). PCR reaction products were cloned using the TOPO® Cloning Kit for Sequencing (Invitrogen), and plasmid DNA was prepared from independent bacterial colonies, normally 4 per PCR product. The purified DNA was sequenced using the Applied Biosystems 3100 or 3730 Genetic Analyzer. All sequences containing mutations and select sequences with no mutations were re-sequenced for verification. Mutations were identified using the NCBI BLAST program against WNV genome accession sequence M12294. Treated and untreated samples were run in parallel to control for the background mutation frequency of RT-PCR, and samples sizes were balanced as much as possible. Mutation frequencies between control and ribavirin-treated sequences were compared statistically with Pearson's chi-square exact test statistic (StatXact-3 for Windows Version 3.1, CYTEL Software Corporation 1997) with nucleotides (nts) treated as independent. Since 93% (52/56) of the observed mutations were found to be unique, clone selection was considered sufficiently independent for the analysis.

2.7. Relative infectivity of vRNA

To assess the effect of the drug on the infectivity of WNV, we compared infectious virus and viral RNA titers from cell supernatants with varying doses of ribavirin. HeLa cells were prepared in 24-well plates as described above with 0, 15, 32, and 100 μg/mL ribavirin. After overnight incubation, triplicate wells for each dose were infected with an MOI of 0.01 (5000 $CCID_{50}$) of WNV and incubated 3 days. The supernatant was removed from the cells and tested in duplicate for infectious virus using the $CCID_{50}$ assay described above. RNA from 100-μL aliquots of the supernatant was extracted using the RNeasy Mini Kit™ (Qiagen, Valencia, CA). Exogenous rat liver RNA was added to each supernatant before RNA extraction, and a separate qRT-PCR assay was run to demonstrate equivalent recovery of rat GAPDH mRNA between samples. This showed that RNA extraction did not introduce important variation into the assay. Each RNA sample was then titered in triplicate using the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) described by Shi et al. (2001).

Viral RNA was quantified using the Brilliant™ qRT-PCR One-Step Core Reagent Kit (Strategene, Cedar Creek, TX) with StrataScript RT polymerase for cDNA synthesis and SureScript Taq polymerase for PCR. Primers were selected from the E protein region of the WNV genome; forward primer 5′-tcagcgatctctccaccaaag, position 1160–1180; reverse primer 5′-gggtcagcacgtttgtcattg, position 1229–1209.

Table 1
Anti-WNV activity and toxicity of ribavirin in various cell lines infected with WNV

Cell line	MOI[a]	Day 2 EC_{90}[b]	Day 6 EC_{90}[b]	Day 6 IC_{50}[c]	SI[d]
HeLa	$10^{-3.4}$	7.7 ± 0.8	9.5 ± 3.9	>1000	>106
L929	10^{-5}	11.6 ± 3.0	28.1 ± 26.4	>570	>20
MA-104	10^{-5}	9.8 ± 5.0	129 ± 123	>1000	>7.8
Vero	10^{-5}	31.6 ± 13.5	329 ± 230	>1000	>3.0
CV-1	10^{-5}	56.9 ± 29.6	>414	>1000	>2.4

[a] Multiplicity of infection = approximate $CCID_{50}$ of WNV infected/cells plated.
[b] Effective concentration (μg/mL) to inhibit virus yield by 90%.
[c] 50% inhibitory concentration (μg/mL) by drug-induced, visual CPE in uninfected cells.
[d] Selectivity index = IC_{50}/EC_{90} on day 6.

The probe was 5′-tgcccgaccatgggagaagctc, position 1186–1207, with a 5′ reporter, 6-carboxyfluorescein (FAM), and a 3′ quencher, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA). Primer concentrations were 0.8 μM and the probe concentration was 0.2 μM. A 2 μL aliquot of sample RNA eluate was used for each reaction. The qRT-PCR was performed on the Opticon2™ system (M.J. Research, Waltham, MA). Thermal cycling consisted of 50 °C for 30 min for RT, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Shi et al., 2001). WNV RNA standards for qRT-PCR were prepared by in vitro transcription of target DNA using the T7 mMessage mMachine Kit™ (Ambion, Austin, TX). The RNA product was then quantified by 260/280 nm spectrophotometry, and serial dilutions of standard WNV RNA from 10^4 to 10^9 genome equivalents (ge)/μL were tested in triplicate in parallel with the test samples. The standard curve had an R^2 value of 0.988. The average of three RNA titers was used in calculations for each sample, and RNA titers were converted to genome equivalents per 100 μL of supernatant for comparison to $CCID_{50}$ titers. The infectious ratio was calculated by dividing infectious virus per μL by ge per μL. Differences were evaluated using Student's *t*-tests (JMP™ statistical software).

3. Results

3.1. Antiviral efficacy and toxicity of ribavirin

Ribavirin appeared effective against the low-level inoculum of WNV after 2 days in five cell lines, but this was sustained for 6 days in only HeLa cells (Table 1). The sustained effect was considered a predictor of selective antiviral activity rather than metabolic inhibition of the cell. The EC_{90} results for ribavirin in some cells after 6 days had relatively high standard deviations (S.D.), which was considered as part of the criteria for selecting the cell line for additional testing. In the subsequent experiments with 5000 $CCID_{50}$ of WNV and 100 μg/mL ribavirin, ribavirin effectively reduced WNV by 5 $\log_{10}$ ($P < 0.001$) compared to untreated controls (Fig. 1). The difference in virus yield for ribavirin-treated versus untreated controls was 1 $\log_{10}$ or less in the other cell lines (Fig. 1). Since ribavirin was more effective in HeLa cells, they were selected for use in the sequencing and infectivity studies. The 50% inhibitory concentration (IC_{50}) of ribavirin observed by visual CPE was >570 μg/mL in L929 cells and >1000 μg/mL in the other cell lines tested, well above the EC_{90} values observed (Table 1). A cytostatic effect of ribavirin in HeLa cells was observed visually in the 6-day EC_{90} study at doses above 320 μg/mL, and the 50% inhibitory concentration (IC_{50}) of ribavirin was 212 μg/mL in HeLa cells using the neutral red assay. Regression analysis predicted that 47 μg/mL of ribavirin, the dose used for the HeLa cell viral extinction study, would cause 20% inhibition of NR uptake in HeLa cells.

3.2. Viral extinction

Lethal mutagenesis caused by accumulated mutations from successive rounds of replication (Loeb et al., 1999) was evaluated by treating cells with ribavirin below the observable toxic dose, infecting cells with WNV, and then sequentially passing the resulting virus repeatedly to fresh cells. WNV was consistently driven to extinction by passing virus sequentially in HeLa cells with 47 μg/mL ribavirin, while control samples showed high virus titers (>10^7 $CCID_{50}$/0.1 mL) after each passage (Fig. 2A). A repeat study in HeLa cells using a 1/4 dilution between each passage yielded similar results with no detectable virus after 5 passages, showing that the

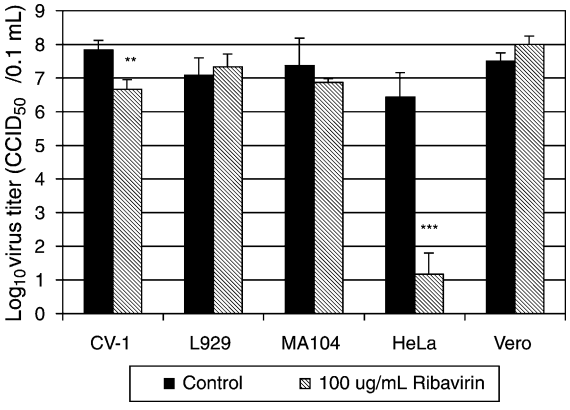

Fig. 1. Mean virus titers ($CCID_{50}$/100 μL ± S.D.; $n = 3$) from cells pretreated with 100 μg/mL ribavirin and infected with 5000 $CCID_{50}$ of WNV and incubated 3 days ($^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

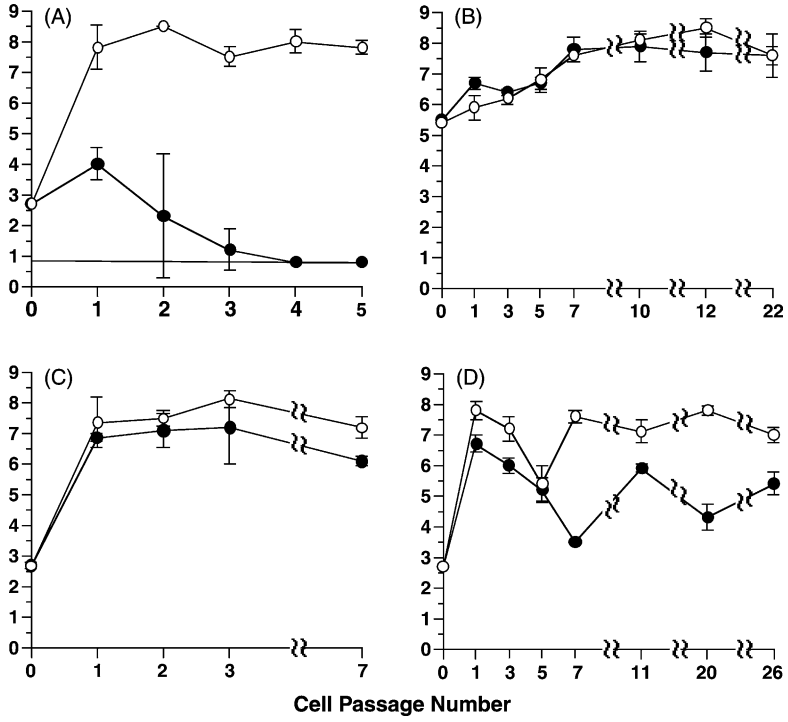

Fig. 2. Mean WNV titers ± S.D. after sequential passage in cells with ribavirin (●) or controls without ribavirin (○). The limit of detection was $10^{0.8}$ $CCID_{50}$/0.1 mL. (A) HeLa cells treated with 47 μg/mL ribavirin ($n = 6$). (B) L929 cells with 100 μg/mL ribavirin ($n = 3$). (C) MA-104 cells with 100 μg/mL ribavirin ($n = 3$). (D) CV-1 cells with 100 μg/mL ribavirin ($n = 3$).

1/100 dilution did not eliminate the virus. In contrast, the virus was passed sequentially in three other cell lines treated with 100 μg/mL ribavirin, and the virus population continued to reach high numbers through many passages. In L929 cells treated with 100 μg/mL ribavirin, WNV replicated to $>10^6$ infectious doses/0.1 mL repeatedly for 22 passages (Fig. 2B). Likewise in MA-104 cells viral titers were approximately equal to controls for 7 passages (Fig. 2C). In CV-1 cells, passages 1 through 7, 11, 20 and 26 were titered; the virus titer was 10- to 100-fold lower than that of untreated controls, but in each passage the virus reached at least 10^4 $CCID_{50}$/0.1 mL with no indication of eventual viral extinction (Fig. 2D).

3.3. Sequence mutations

A low dosage of ribavirin (15 μg/mL) caused error-prone replication in the WNV molecular clone (Table 2). The transition mutation frequency for ribavirin-treated sequences was 0.703 per 1000 nucleotides (nts), significantly higher than 0.345 per 1000 nts for control sequences ($P < 0.05$). The increased mutation frequency was greater when only C to U and G to A transitions were considered, 0.567 per 1000 nts with ribavirin compared with 0.173 per 1000 control nts ($P < 0.01$). In contrast, there were no statistical differences in transversion frequencies between control samples and treated samples. G to A transitions were the predominant mutations in ribavirin-treated samples, and C to U transversions were the second most common mutations (Table 3).

3.4. Relative infectivity of viral RNA

Viral RNA isolated from cell supernatant after 3 days of incubation with treated and untreated HeLa cells was titered using quantitative RT-PCR, and infectious virus was titered

Table 2
Mutations in sequences from WNV propagated in HeLa cells with 15 μg/mL ribavirin

Mutation type	Number of mutations[a]		Frequency per 1000 nucleotides sequenced[a]	
	Ribavirin	Control	Ribavirin	Control
All mutations	34	22	0.771	0.475
Transitions	31*	16	0.703	0.345
C-U or G-A	25**	8	0.567	0.173
Transversions	3	6	0.068	0.129

[a] Out of 44,089 ribavirin-treated and 46,349 control nucleotides sequenced.
* $P < 0.05$.
** $P < 0.01$.

Table 3
Numbers of the different substitution mutations observed in WNV after 3 days growth in HeLa cells with 15 μg/mL ribavirin

	Transitions				Transversions							
	A-G	C-U	U-C	G-A	A-C	A-U	C-A	C-G	U-A	U-G	G-C	G-U
Ribavirin[a]	4	6	2	19*	0	1	0	0	2	0	0	0
Control[a]	2	2	6	6	1	0	0	0	1	4	0	0

[a] Mutations out of 44,089 ribavirin-treated and 46,349 control nucleotides sequenced.
* $P < 0.05$.

Table 4
Relative infectivity of West Nile virus RNA detected by quantitative RT-PCR from HeLa cell supernatants after 3 days incubation with or without ribavirin (MOI ≈0.01)

Ribavirin (μg/mL)	RNA titer[a]	Infectious virus[b]	Percent genome infectivity[c] (%)
0	8.0 ± 0.04	7.8 ± 0.26	62.5
15	6.9 ± 0.08	6.2 ± 0.19	18.9
32	4.7 ± 0.15	2.9 ± 0.38	1.6***

[a] Log_{10} of genome equivalents per 0.1 mL ± S.D.
[b] Log_{10} of 50% cell culture infectious doses ($CCID_{50}$) per 0.1 mL ± S.D.
[c] Percent of genome equivalents detected that were infectious.
*** $P < 0.001$ compared to untreated control.

on the same samples. Both infectious virus and viral RNA decreased with increasing doses of ribavirin, but reduction in infectious virus was greater than reduction of viral RNA (Table 4). No infectious virus was detected with 100 μg/mL. A 32 μg/mL concentration of ribavirin reduced infectious virus by 4.9 log_{10} compared to untreated controls, while it decreased the RNA titer by only 3.3 log_{10}. Thus, the proportion of infectious virus to total RNA ratio was 39-fold lower (62.5%/1.6%) in the presence of 32 μg/mL of ribavirin compared with untreated controls ($P < 0.001$). Studies on the intracellular viral RNA also demonstrated a similar reduction in infectivity in the presence of ribavirin (data not shown).

4. Discussion

Since RNA viruses replicate with high genetic variation, they presumably exist close to the "error-threshold," where increased mutations may lead to reduced fitness or viral extinction (Domingo, 2000). Experiments with poliovirus have previously shown evidence that this threshold is effectively crossed by treating with ribavirin (Crotty et al., 2001). Even relatively low numbers of mutations can accumulate through multiple rounds of replication and lead the viral population to extinction (Loeb et al., 1999). Data in this study showed extinction of WNV in HeLa cell culture after 4 passages with 47 μg/mL ribavirin. The increase in virus titer after the first passage, followed by a decrease in subsequent passages (Fig. 2A) in HeLa cells, suggested that a single passage did not yield enough mutations to decrease the viral population, but that accumulation of mutations over several passages led to an antiviral effect. Failure of ribavirin to extinguish the virus in the other cell lines was likely because phosphorylation of ribavirin to ribavirin triphosphate (RTP) differs between cell lines, which has been demonstrated previously (Smee et al., 2001). If ribavirin induced error-prone replication of WNV in the other cells, it was not sufficient to extinguish the populations. The cell-dependent activity of ribavirin against WNV may partly account for why ribavirin did not prevent WNV disease in patients (Hrnicek and Mailliard, 2004) or improve disease outcome in hamsters (Morrey et al., 2004) or in BALB/c mice (unpublished data).

This data showed that ribavirin caused error-prone replication of WNV, since the frequency of transition mutations was higher in the ribavirin-treated samples than in controls. The number of C to U and G to A mutations, along with the absence of A to G and U to C mutations (Table 3) indicated that incorporation of ribavirin by WNV RdRp was likely as a guanosine analog rather than as an adenosine analog. We proposed that after RTP incorporation into the genome, promiscuous base pairing occurred in subsequent replication wherein RTP paired with equal frequency to a uridine or a cytidine (Crotty et al., 2000), leading to increased C to U and G to A mutations. This mechanism was supported by the mutations observed (Table 3). It was also notable that more G to A mutations were observed (19) than C to U mutations (6) in ribavirin-treated sequences, suggesting that ribavirin was more prone to incorporate into the genome itself rather than the negative strand intermediate. This mechanism seemed likely in light of the types of mutations observed and previous findings that ribavirin was incorporated into other viral genomes (Crotty et al., 2000). Another possible mechanism that would explain the specific types of mutations observed in this study would be a direct result of IMPDH inhibition that may force nucleotide misincorporation by the viral polymerase in the absence of sufficient intracellular GTP.

Since ribavirin has multiple modes of action, it is difficult to isolate the proportion of the antiviral activity that was due to lethal mutagenesis. Ribavirin normally decreases intracellular GTP pools by inhibiting IMPDH (Streeter et al., 1973).

Recent studies show that ribavirin reduces intracellular GTP levels more in HeLa cells than in Vero cells (Leyssen et al., 2005), which correlates with the difference in antiviral efficacy observed between cell lines. IMPDH inhibitors such as mycophenolic acid have demonstrated activity against WNV (Morrey et al., 2002), and we were able to reverse the antiviral effect of ribavirin by addition of guanosine at a concentration approximately 10-fold higher than that of ribavirin (data not shown). Therefore, GTP depletion by ribavirin may work in concert with error-prone replication by increasing the probability of ribavirin incorporation into the genome. The ratio of infectious virus to viral RNA in cell supernatants decreased with increasing doses of ribavirin, suggesting a direct effect on nascent virions which is likely due to ribavirin-induced mutations.

These studies were conducted using low ribavirin doses that caused no visible, drug-induced CPE in the subconfluent monolayers of cells used. However, the drug has been shown to have cytostatic effects in other studies (Rankin et al., 1989; Muller et al., 1977). The relatively low inhibition of neutral red uptake by ribavirin-treated HeLa cells in this study suggested that the logarithmic reduction in viral titers observed in the viral extinction assay was due to antiviral effects and not cellular toxicity by the drug. A relatively low concentration of ribavirin (15 μg/mL or 61.5 μM) was necessary to allow propagation of sufficient viral RNA for sequencing studies. It was predicted that higher ribavirin doses would induce proportionately more mutations if sufficient competent virus could be produced in the presence of such doses. For example, the same types of mutations have been shown to be induced by RTP in poliovirus, but at a much higher frequency using 1000 μM ribavirin (Crotty et al., 2000). Furthermore, the mutation frequency demonstrated here was considered a minimum representation, since genomes that incur higher numbers of mutations would likely fail to replicate, and therefore go undetected.

Ribavirin was effective in preventing WNV replication in HeLa cells at subtoxic doses, and led to extinction of a viral population after multiple passages. However, it was not effective in four other cell lines or in WNV-animal models (Morrey et al., 2004), and would not, therefore, be a candidate of choice for antiviral therapy against WNV disease. The data showed that low doses of ribavirin increased the frequency of mutations in the viral genome, and that ribavirin decreased the relative infectivity of WNV RNA. These data support the hypothesis that ribavirin may cause error catastrophe as one of its modes of action against West Nile virus.

Acknowledgements

We thank Aaron Brown, Grant Roper, Cary Jaques, and John R. Haynie for technical assistance, and Susan Durham for statistical analysis. This work was partially supported by Contracts NO1-AI-65291 and NOI-AI-15435 from the Virology Branch, NIAID, NIH.

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