The Anti-Yellow Fever Virus Activity of Ribavirin Is Independent of Error-Prone Replication

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ABSTRACT

The precise mechanism by which the broad-spectrum anti-RNA virus agent ribavirin elicits its in vitro antiviral effect has remained a matter of debate. We have demonstrated that inhibition of cellular inosine monophosphate dehydrogenase (IMPDH) activity, and thus depletion of intracellular GTP pools, is the predominant mechanism by which ribavirin inhibits the replication of four flavi- and two paramyxoviruses (*J Virol* **79**: 1943–1947, 2005). As a consequence, induction of error catastrophe, which has been proposed as a mechanism by which ribavirin may elicit its anti-RNA virus activity, may be expected to have little, if any, impact on its antiviral effect. The flavivirus yellow fever virus (17D vaccine strain) was cultured for five consecutive passages in the presence of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), 5-ethynyl-1-β-D-ribo-furanosylimidazole-4-carboxamide (EICAR) (the 5-ethynyl analog of ribavirin), or mycophenolic acid (MPA; a compound that exclusively inhibits IMPDH). The reduction in infectious virus yield brought about by ribavirin (as well as MPA and EICAR) was paralleled by a similar reduction in viral RNA yield; in case of error-prone replication, the infectious virus yield is expected to decrease significantly faster than the viral RNA yield. In addition, pre-extinction populations of the virus that has suffered a maximum impact of treatment with ribavirin did not accumulate an increased number of mutations. Very similar observations were obtained with EICAR and with MPA, a molecule that cannot be incorporated into viral RNA. These data thus allow us to conclude that the in vitro anti-yellow fever virus activity of ribavirin is independent of error-prone replication.

The synthesis and antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was first reported in the early seventies (Sidwell et al., 1972; Witkowski et al., 1972). At the outset, inosine 5'-monophosphate (IMP) dehydrogenase, a cellular enzyme that converts IMP to xanthosine 5'-monophosphate in the de novo synthesis pathway of GMP, was shown to be inhibited by ribavirin 5'-monophosphate (Streeter et al., 1973). The subsequent depletion of intracellular GTP pools, thus compromising the supply of this nucleotide for the synthesis of progeny viral RNA, provided a reasonable explanation for the broad-spectrum anti-RNA virus activity of ribavirin. Ribavirin-resistant Sindbis virus was shown to contain mutations in the viral guanylyl-transferase gene that increased the affinity of this enzyme for GTP. As a consequence, the drug-resistant Sindbis virus was shown to replicate efficiently in cells with low intracellular GTP concentrations, either induced by ribavirin or by other IMP dehydrogenase inhibitors (Malinoski and Stollar, 1981; Scheidel and Stollar, 1991).

Later, several other mechanisms were reported that may contribute to the antiviral activity of ribavirin. These are inhibition of 1) vaccinia virus guanylyltransferase by ribavirin-5'-triphosphate (TP) (Goswami et al., 1979; Bougie and Bisaillon, 2004), 2) influenza virus RNA-dependent RNA polymerase by ribavirin-5'-TP (Eriksson et al., 1977), 3) reovirus helicase by ribavirin 5'-TP (Rankin et al., 1989), and 4) dengue virus methyltransferase by ribavirin 5'-TP (Benarroch et al., 2004). Poliovirus RNA-dependent RNA polymerase was shown to incorporate ribavirin opposite to cytidine and uridine (Crotty et al., 2000), and poliovirus, which was cultured in the presence of high concentrations of ribavirin (1 mM), accumulated 10-fold more mutations in its genome than virus grown in untreated cultures (Crotty et al., 2001). Accumulation of (a number of) lethal mutations was shown to result in reduced infectivity of the poliovirus population, a process called error catastrophe

ABBREVIATIONS: IMP, inosine 5'-monophosphate; TP, triphosphate; IMPDH, cellular inosine monophosphate dehydrogenase; WNV, West Nile virus; EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide; YFV, yellow fever virus; $CCID_{50}$, cell culture infective dose 50; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MPA, mycophenolic acid; nt, nucleotide(s); CPE, cytopathic effect; HCV, hepatitis C virus; VC, virus control.

(Crotty et al., 2001). A single mutation (G64S) in the poliovirus RNA-dependent RNA polymerase gene was found to increase the fidelity of the enzyme and to render the virus less susceptible to the mutagenic effect of ribavirin (Pfeiffer and Kirkegaard, 2003).

It remains unclear to what extent each of these different mechanisms contributes to the actual antiviral effect of ribavirin. We have demonstrated that depletion of intracellular GTP pools in cell culture caused by ribavirin closely correlates with inhibition of the replication of (four different) flaviviruses (and two paramyxoviruses) (Leyssen et al., 2005). Day and colleagues recently presented evidence that both inhibition of the IMPDH and error-prone replication contribute to the antiviral effect of ribavirin against West Nile virus (WNV) replication in HeLa cells (Day et al., 2005). The present study was designed to explore whether error-prone replication contributes to the in vitro antiviral activity of ribavirin against the yellow fever virus (YFV).

Materials and Methods

Compounds, Cells, and Virus. Ribavirin (Virazole) was purchased from Valeant Pharmaceuticals International (Costa Mesa, CA), EICAR was kindly provided by Dr. A. Matsuda (Hokkaido University, Sapporo, Japan), and mycophenolic acid [6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid] was purchased from Sigma (Bornem, Belgium). Vero (African green monkey kidney) cells were used throughout the experiments. Yellow fever virus 17D vaccine strain (Stamaril; Aventis Pasteur MSD, Brussels, Belgium) was passaged once in Vero cells to prepare a working virus stock and was stored at $-80°C$ until used.

Titration for Infectious Virus Content. One-day-old confluent Vero cell monolayers grown in 96-well trays were infected with 5-fold serial dilutions of supernatant samples harvested at 5 days postinfection from infected cell cultures. Each titration was performed in sextuplicate. After incubation for 6 days at 37°C (5% CO_2 atmosphere, 99% relative humidity), cells were fixed with 70% ethanol and stained overnight with 2% Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany). Cultures were examined microscopically for cytopathic effects. The 50% cell culture infective dose ($CCID_{50}$) was determined according to the method of Reed and Muench (1938). The detection limit of this method was determined to be 1.25 $CCID_{50}$/ml.

Quantitation of Viral RNA Load. Total RNA was extracted from 150 μl of cell culture supernatant according to the manufacturer's instructions (NucleoSpin RNA Virus kit; Machery-Nagel, Düren, Germany). A one-step quantitative RT-PCR was performed in a 25-μl reaction mix (One-Step Reverse Transcriptase qPCR Master Mix; Eurogentec, Seraing, Belgium) using the following primers and probe: forward primer (900 nM final concentration), 5'-TGG CAT ATT CCA GTC AAC CTT CT-3'; reverse primer (900 nM), 5'-GAA GCC CAA GAT GGA ATC AAC T-3'; and 5-carboxyfluorescein-MGB probe (200 nM), 5'-TTC CAC ACA ATG TGG CAT G-3' on an SDS7000 (Applied Biosystems, Lennik, Belgium). The standard curve for absolute quantitation of YFV RNA consisted of a 10-fold dilution series of a template sample with known quantity. Data are expressed in RNA copies per milliliter. The detection limit of the RT-qPCR in this experimental setup was determined to be 111 RNA copies/ml.

Serial Passage of YFV in the Presence of Ribavirin, EICAR, and MPA. Vero cells were seeded in 96-well trays in cell growth medium (minimum Eagle's medium, 10% heat-inactivated fetal calf serum, 0.29 mg/ml glutamine, and 0.075% bicarbonate). Twenty-four hours later, medium was removed from the confluent monolayers and replaced by 100 μl of assay medium (minimum Eagle's medium, 2% FCS, 0.29 mg/ml glutamine, and 0.075% bicarbonate) either or not containing serial dilutions of compound at a 2 times concentration. Subsequently, the cultures were infected with 100 μl of virus inoculum ($\sim$1.350 $CCID_{50}$/well) of the reference virus. Each treatment condition was carried out in quadruplicate on two separate 96-well trays per experiment and in two independent experiments. After a 1-h incubation period, culture supernatant was removed. The monolayers were washed three times with warm assay medium to remove nonadsorbed virus. Then, cultures were further incubated in the absence or presence of serial dilutions of the compounds for 5 days. At 5 days postinfection, the first signs of cytopathic effect became visible in the virus control (VC) condition, and the infectious virus yield in the culture supernatant was at that time comparable with that of the original virus inoculum. The supernatant of four replicate wells per plate was collected and pooled; 150 μl was stored at $-80°C$ for later use in RT-qPCR, 6 × 25 μl was immediately used for titration for infectious virus content, and 20 μl was used as inoculum for the subsequent serial passage (at a dilution of 1/1000) in the absence or presence of compound. The remaining volume was stored at $-80°C$ for sequencing. The entire protocol was repeated for five successive passages.

Statistical Analysis. The ratio of $CCID_{50}$/ml to RNA copies per milliliter was compared among the four conditions (virus control, ribavirin, EICAR, and MPA) using a Kruskal-Wallis test. Pairwise comparison of these data sets was performed using the Wilcoxon test. A linear regression model was used to assess whether a correlation exists between RNA copies per milliliter and $CCID_{50}$/ml for the four conditions. All statistical tests were performed using SAS software version 9 (SAS Institute, Tervuren, Belgium).

Mutation Analysis. Total RNA was extracted from the culture supernatant, and viral RNA yield was quantified using RT-qPCR. Samples were selected based on the following criteria: 1) the virus in the sample should have been replicating for at least one passage in the presence of compound, 2) the virus should be undetectable (by RT-qPCR or titration) after one extra passage in the presence of the compound (=pre-extinction virus population), and 3) an equivalent sample (i.e., same passage number) should be available for all three of the treatment conditions. The RNA template concentration was normalized so that an identical RNA input (1000 RNA copies) was used for the subsequent RT-PCR reaction. The RT-PCR reaction was performed using the One-Step RT-PCR kit (QIAGEN, Leusden, The Netherlands) in a 50-μl reaction mix: 14 μl of water, 10 μl of 5× buffer, 2 μl of dNTP, 2 μl of Enzyme mix, 1 μl of RNasin (Promega, Leiden, The Netherlands), 0.5 μl of forward primer (60 μM; 5'-CTG TGA GCT TCC ATG GTA GTG AT-3'), 0.5 μl of reverse primer (60 μM; 5'-CAG AGA TGG AGA CTC ATA CTA C-3'), and 20 μl of RNA template. The following thermocycling profile was used: 30 min at 50°C, 15 min at 95°C, 30 cycles of 20 s at 94°C, 30 s at 55°C, 150 s at 72°C (extended by 5 s per cycle), and final extension for 10 min at 72°C. Because of the very low RNA template input, no amplicon (expected to be 2.591 nt) was visible after agarose gel electrophoresis. Therefore, the one-step RT-PCR reaction was used as template (5 μl) for an inner PCR using the AccuPrime kit (Invitrogen, Merelbeke, Belgium) in a 50-μl reaction mix: 39.1 μl of water, 5 μl of 10× buffer, 0.25 μl of inner forward primer (60 μM; 5'-CAA GGA CAC CTC CAT GCA GAA GA-3'), and 0.25 μl of inner reverse primer (60 μM; 5'-CCT AAC TTA CAG GAT GTT GGA ACC-3'). The following thermocycling profile was used: 2 min at 95°C, 30 cycles of 15 s at 95°C, 30 s at 55°C, 80 s at 68°C (extended by 3 s per cycle), and final extension for 10 min at 68°C. The amplified 1.344-nt fragment was purified by gel extraction (QIAquick gel extraction kit; QIAGEN). After A-tailing with SuperTaq (Sphaero Q, Gorinchem, The Netherlands) for 15 min at 72°C, the fragment was cloned into pCR-TOPO (TOPO cloning kit for sequencing; Invitrogen), and transformed into One Shot chemically competent cells. For each of the selected samples, at least 23 positive clones were selected, and plasmid DNA was extracted using the Wizard Plus Miniprep DNA purification system (Promega). Plasmid inserts were sequenced using the forward and reverse primer designed for the RT-qPCR described above (BigDye

Terminator Cycle Sequencing kit; Applied Biosystems). Sequencing data were processed using Vector NTI software (Invitrogen).

Results

Effect of Antiviral Drugs on Viral Replication during Successive Passage of YFV. YFV was allowed to replicate for five consecutive passages in the presence of 2.4 (data not shown), 12, or 60 μg/ml ribavirin (Fig. 1, A and D). None of these concentrations of ribavirin resulted in a reduction of infectious virus or viral RNA yield compared with the untreated virus control condition (Fig. 1, A and D). Replication of YFV in the presence of ribavirin at concentrations of 120, 180, 240, or 300 μg/ml resulted in a dose-dependent decrease of infectious virus yield and viral RNA load (Fig. 1, A and D). Consecutive passages of virus in the presence of the drug at the indicated concentrations (120, 180, 240, or 300 μg/ml) ultimately resulted in viral extinction. The higher the concentrations of compound used, the fewer passages were needed to completely abolish viral replication.

Comparable observations were made with EICAR and MPA. A concentration of 2.5 μg/ml EICAR resulted in complete extinction of viral replication at the fifth passage; at a 5-fold higher concentration (12.5 μg/ml), viral replication was cleared after three passages (Fig. 1, B and E). As little as 1.25 μg/ml MPA was needed to abolish viral replication in three passages (Fig. 1, C and F). Overall, MPA proved to be more potent than EICAR, which in turn was more effective than ribavirin in clearing virus replication. When the effects of the respective compounds on both viral RNA yield and infectious virus yield were compared, almost identical profiles were observed for each compound at any particular concentration.

Statistical Analysis of the Effect of Treatment on YFV Viability. For each single sample of the experiments depicted in Fig. 1 (A–F), the infectious virus titer ($CCID_{50}$ per milliliter) and viral RNA yield (RNA copies per milliliter) was quantified. In such case that a compound would cause error-prone replication, the ratio of infectious virus yield to viral RNA yield is expected to be lower than this ratio in culture supernatant samples obtained from untreated infected cultures.

For the untreated control cultures as well as for each of the treated cultures, the $\log_{10} CCID_{50}$/ml was plotted against the corresponding $\log_{10}$ RNA copies per milliliter (Fig. 2). Linear correlations were obtained [virus control: $R^2 = 0.938$ (Fig.
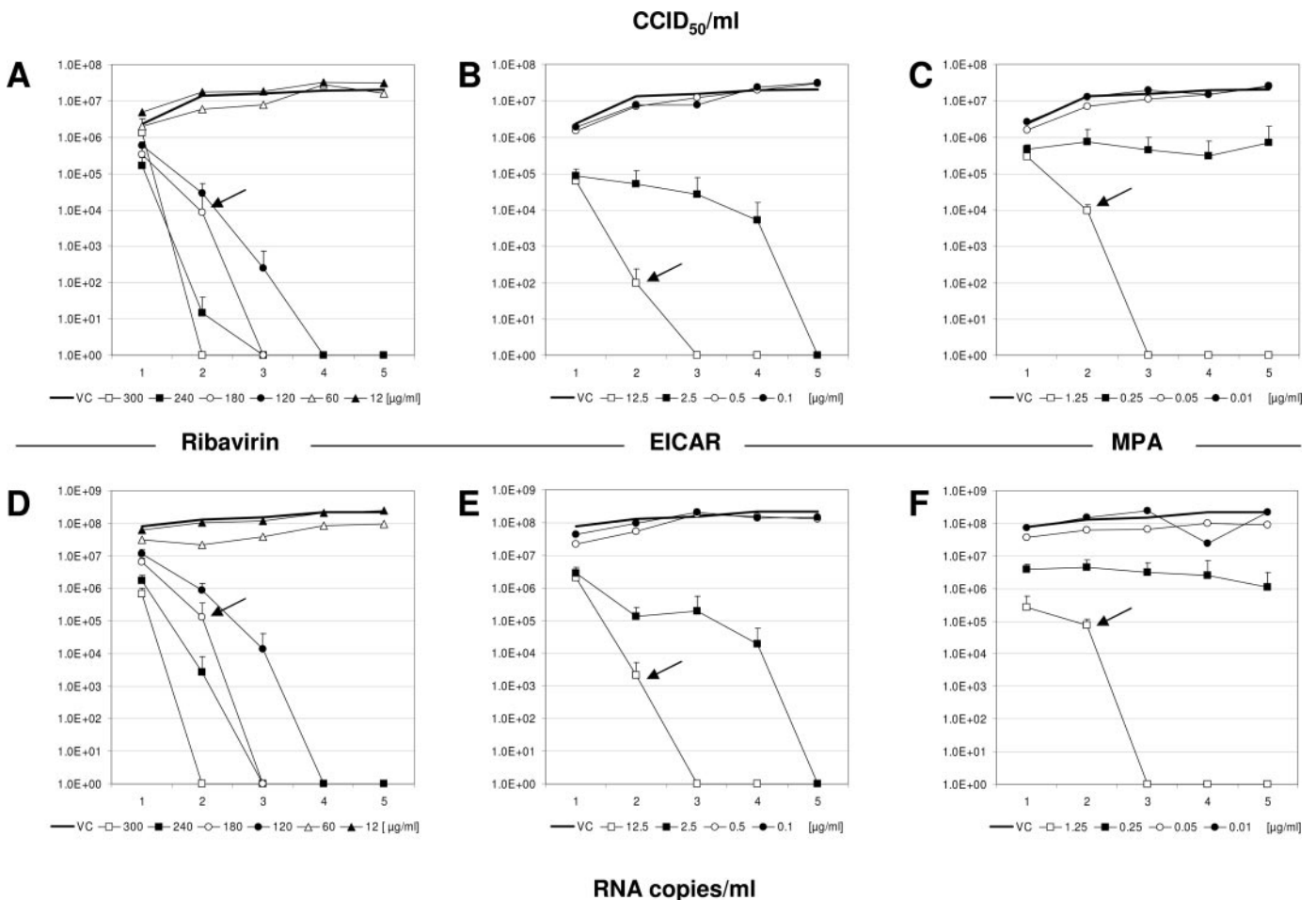

Fig. 1. Effect of ribavirin, EICAR, and MPA on YFV yield during serial passage under different treatment conditions in Vero cells. Quantity of infectious YFV virus (A–C; presented as $CCID_{50}$ per milliliter; y-axis) as determined by titration for infectious virus content. Quantity of YFV RNA (D–F; presented as RNA copies per milliliter; y-axis) as assessed by RT-qPCR. Samples of cell culture supernatant were collected at 5 days postinfection from infected Vero cells treated with different concentrations of ribavirin (A and D), EICAR (B and E), or MPA (C and F). The yield data are presented as a function of the passage number (1–5; x-axis). Values represent the average of two to four independent experiments. For enhanced readability, standard deviations are only shown for curves deviating significantly from the curve obtained for the virus control (VC) condition. Arrows indicate samples that were selected for mutation analysis (see Tables 1–3).

2A); ribavirin: $R^2 = 0.901$ (Fig. 2B); EICAR: $R^2 = 0.880$ (Fig. 2C); and MPA: $R^2 = 0.889$ (Fig. 2D)]. When subanalyses were made, it was evident that these correlations were not dependent on the passage number at which the samples were collected or on the concentration of compound at which the virus was cultured (data not shown).

To allow detailed statistical analysis, the ratio $CCID_{50}$ per milliliter to RNA copies per milliliter was calculated for each supernatant sample. A Kruskal-Wallis test was performed on all data sets simultaneously, and a p value of <0.0001 was obtained, which indicates that a statistically significant difference exists between at least two (not yet specified) data sets. Therefore, a two-sided Wilcoxon test was used for pairwise comparison of the four data sets. A statistically significant difference was calculated when either one of the treatment conditions (ribavirin, EICAR, or MPA) was compared with the untreated virus control ($p = 0.0003$, 0.0005, and <0.0001, respectively). The median of the untreated virus control was 0.029 (ratio $CCID_{50}$ per milliliter to RNA copies per milliliter); for the treatment conditions (ribavirin, EICAR, and MPA, respectively), medians of 0.072, 0.073, and 0.117 were calculated. The different treatment conditions did not differ significantly from each other (data not shown), except for a minimal difference between the MPA and ribavirin condition ($p = 0.016$).

Mutation Analysis of YFV Grown in the Presence of Compound. We next wanted to determine whether virus that has been replicating in the presence of ribavirin (or

EICAR or MPA) contained an increased number of mutations in the genome compared with untreated virus. For each compound, one sample, containing a pre-extinction virus population, was selected for such analysis (the conditions marked with an arrow in Fig. 1). For these conditions, infectious virus yield and viral RNA yield was reduced by at least 2 $\log_{10}$, and one extra passage in the presence of the compound resulted in complete extinction of viral replication.

For each sample, at least 23 different clones were generated and sequenced. This represents a total of more than 30,000 nt per sample (Table 1, A). Furthermore, 1) the total number of mutations compared with the YFV 17D consensus sequence (GenBank no. X03700) (Table 1, B), 2) the number of clones with a unique set of mutations (Table 1, D) as well as 3) the total number of unique mutations (Table 1, E) were determined. In the ribavirin-treated virus sample, but also in the EICAR-treated virus sample, each time two deletion mutants were detected, which were different for both conditions (data not shown).

The frequency of the total number of mutations per 1000 nt (Table 1, C) as well as that of unique mutations per 1000 nt in unique clones (Table 1, F) was calculated. When the mutation frequencies of the different treatment conditions were compared with that of the in parallel cultured untreated virus (virus control), very comparable results were obtained (Table 1, C and F). Only the EICAR condition (analysis of the total number of mutations; Table 1, C) showed a 1.14-fold increase in mutation frequency. The mutation frequency of
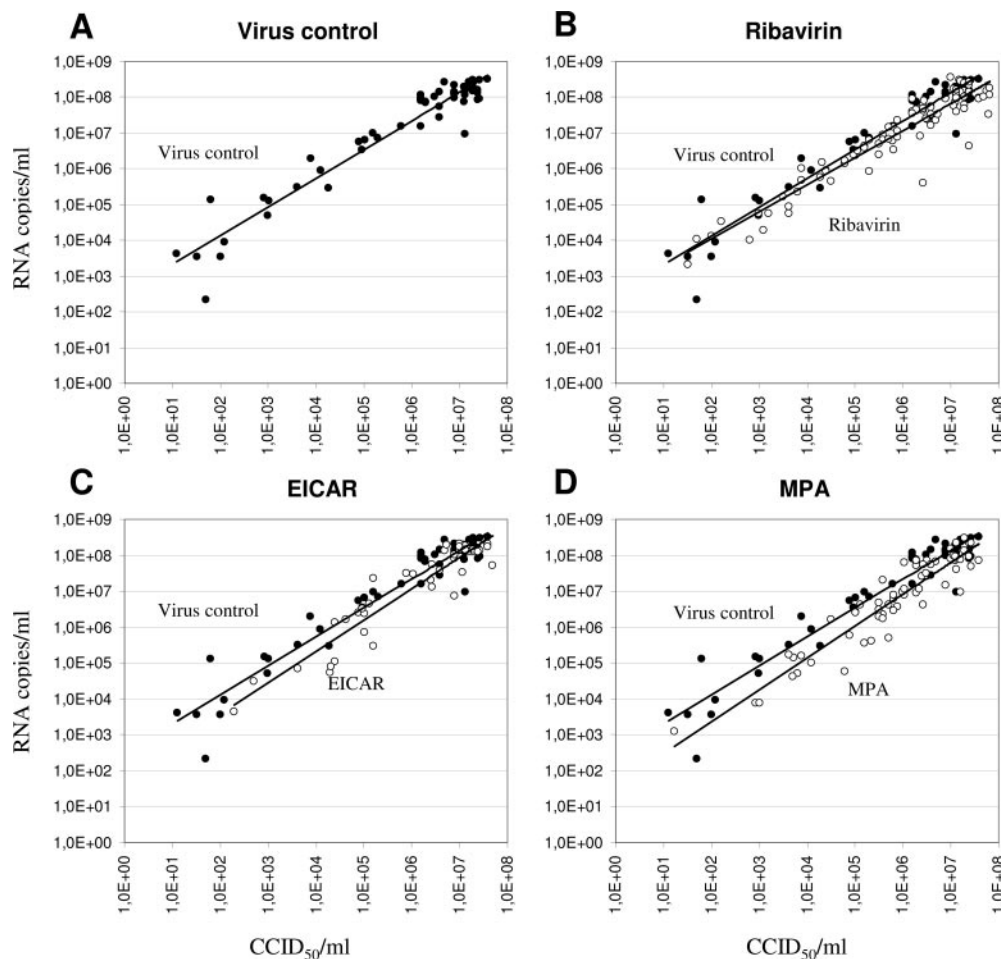

Fig. 2. Correlation of infectious virus yield with viral RNA yield. Correlation of the $\log_{10}$ $CCID_{50}$ per milliliter (x-axis), as determined by titration for infectious virus content, with the $\log_{10}$ RNA copies per milliliter (y-axis), as determined by RT-qPCR, for VC (●) (58 data points, $R^2_{VC} = 0.94$) (A), VC (●) compared with ribavirin (○) (92 data points, $R^2_{ribavirin} = 0.90$) (B), VC (●) compared with EICAR (○) (56 data points, $R^2_{EICAR} = 0.88$) (C), and VC (●) compared with MPA (○) (65 data points, $R^2_{MPA} = 0.89$) (D). Data points have been derived from the experiment depicted in Fig. 1.

all other treatment conditions was lower than that of the untreated virus control.

For all samples, the most prevalent mutations were A to G, U to C, C to U, and G to A (Table 2). In particular C-to-U and G-to-A mutations were reported to occur with increased frequency (10- to 13.6-fold increase) in the genome of poliovirus that was allowed to replicate in the presence of ribavirin (Crotty et al., 2000). Therefore, we calculated the frequency per 1000 nt of C-to-U and G-to-A mutations, and, for comparison, also the frequency per 1000 nt of A-to-G and U-to-C mutations (Table 3). The frequency of C-to-U mutations was 2.4-fold higher for the ribavirin condition than for the virus control condition (Table 3). However, this value was similar to the value (2.3-fold increase) obtained for virus grown in the presence of MPA, a molecule that cannot be incorporated into the viral genome. No marked increase in G-to-A mutation frequency was noted for the ribavirin (and EICAR) condition compared with either the untreated (virus control) or MPA condition. Likewise, no marked differences were observed between C-to-U and G-to-A mutation frequencies compared with A-to-G and U-to-C mutation frequencies for each of the compounds.

Discussion

Depending on the virus studied, ribavirin, a compound that is extensively used in the clinical setting, has been suggested to exert its antiviral activity through various mechanisms (Eriksson et al., 1977; Goswami et al., 1979; Rankin et al., 1989; Benarroch et al., 2004; Bougie and Bisaillon, 2004). A few years ago, evidence was provided that ribavirin exerts its activity against poliovirus by induction of an increased mutation frequency (10- to 13-fold) in virus grown in the presence of the drug (Crotty et al., 2001). Ribavirin was shown to base-pair with cytidine and uridine, which was suggested to result in a specific increase in C-to-U and G-to-A mutations (Crotty et al., 2000). Such accumulation of mutations would result in reduced infectivity/viability of the progeny virus, in a process called error catastrophe (Crotty et al., 2001). Error catastrophe has been proposed to be involved in the antiviral activity of ribavirin against Hantaan virus replication (Severson et al., 2003) as well as the replication of foot-and-mouth disease virus (Airaksinen et al., 2003; Pariente et al., 2003), GB virus B (Lanford et al., 2001), and recently also the replication of the flavivirus West Nile virus (Day et al., 2005). However, ribavirin-enhanced lethal mutagenesis was reported not to be the mechanism of action against Sindbis virus (Scheidel et al., 1987; Scheidel and Stollar, 1991) and lymphocytic choriomeningitis virus (Ruiz-Jarabo et al., 2003).

In this study, we examined whether a decrease in infectious YFV production, brought about by ribavirin, was par-

alleled by a concomitant decrease in viral RNA yield. In addition, we investigated whether YFV, when cultured in the presence of concentrations of ribavirin that had a major impact on viral replication, did or did not accumulate significantly more mutations than virus passaged in the absence of the compound. The antiviral effect of ribavirin was compared with that of MPA and EICAR. Mycophenolic acid is a non-nucleoside inhibitor that potently inhibits IMPDH (by interacting with its coenzyme NAD^+) and that has excellent antiflavivirus activity in cell culture. As a non-nucleoside compound, MPA cannot be incorporated into viral RNA. EICAR is the 5-ethynyl analog of ribavirin that is approximately 25-fold more effective in cell culture against flaviviruses than ribavirin (Neyts et al., 1996; Leyssen et al., 2000, 2005). EICAR 5′-monophosphate in turn is approximately 25-fold more potent than ribavirin 5′-monophosphate as an inhibitor of IMPDH (Balzarini et al., 1993, 1998; Leyssen et al., 2005). As a nucleoside analog, EICAR 5′-triphosphate may possibly have (akin to ribavirin 5′-triphosphate) the potential to be incorporated into viral RNA.

Reduction of infectious virus yield by ribavirin was paralleled by a concomitant reduction in total viral RNA load. Similar observations were made for EICAR and for MPA. Ultimately, antiviral treatment with either compound resulted in complete extinction of viral replication. Analysis of the ratios infectious virus to viral RNA yield did not reveal statistically significant differences between the various treatment conditions. Only when the untreated virus control was compared with any of the treatment conditions could a statistically significant difference be calculated. This can be explained because untreated cultures exhibited cytopathic effect (CPE) at the time of collection of supernatant samples, whereas only minimal CPE was observed in ribavirin- and EICAR-treated cell cultures, and no CPE was detectable in the MPA-treated cell cultures. Cell destruction (caused by CPE) results in the release, for example, of immature virus particles. That both the ribavirin- and EICAR-treated cell cultures showed some minor degree of CPE, whereas the MPA-treated cultures did not, may also explain why the *p* values are on the verge of significance when a statistical analysis was performed on the ratios' $CCID_{50}$ per milliliter to RNA copies per milliliter of the ribavirin or EICAR conditions on one hand and the MPA treatment condition on the other hand. Overall, MPA proved to be more potent in clearing YFV replication than EICAR, which in turn was more potent that ribavirin, an order of potency that is also reflected in the ability of the compounds to inhibit virus replication and to cause GTP depletion (Leyssen et al., 2005). None of the three compounds diminished the infectivity of progeny virus.

We next studied whether pre-extinction YFV, propagated

TABLE 1

Mutation analysis

Summary of the sequencing data. The number of sequenced nucleotides is provided in parentheses.

	Virus Control	Ribavirin	EICAR	MPA	
No. of clones sequenced	24 (32256nt)	23 (30912nt)	23 (30912nt)	24 (32256nt)	A
Total no. of mutations	43	40	47	40	B
Mutation frequency/1000 nt	1.33	1.29	1.52	1.24	C
No. of unique clones	12 (16128nt)	13 (17472nt)	16 (21504nt)	9 (12096nt)	D
Total no. of unique mutations	28	26	30	16	E
Mutation frequency/1000 nt	1.74	1.49	1.40	1.32	F

TABLE 2
Distribution of mutations
Distribution of the total number of mutations for each of the different conditions sequenced.
The total number of unique mutations, when different from the total number of mutations, is provided in parentheses.

	A				U				C				G			
	Virus Control	Ribavirin	EICAR	MPA	Virus Control	Ribavirin	EICAR	MPA	Virus Control	Ribavirin	EICAR	MPA	Virus Control	Ribavirin	EICAR	MPA
A to					1	1	2	1	0	5 (2)	0	0	15 (9)	9 (8)	9 (8)	9 (3)
U to	1	2	1	4 (1)					15 (10)	7 (4)	24 (10)	8 (4)	0	0	1	0
C to	0	0	0	0	3 (1)	7 (4)	1	7 (2)		1	1	2 (1)	1	0	0	0
G to	7 (5)	6 (4)	4	9 (4)	0	0	2	0	0	1	1	2 (1)				

in the presence of concentrations of ribavirin that produced an obvious antiviral effect that ultimately resulted in total viral elimination, contained an increased number of mutations. No increase in the overall mutation frequency was observed between treated and untreated virus. At the genomic level, one peculiar difference was noted between the ribavirin/EICAR conditions and the MPA condition; in the latter, no deletion mutants were detected, whereas four deletion mutants were detected in the ribavirin and EICAR conditions (two different deletions each). Others also reported the occurrence of deletion mutants in the genomes of virus treated with ribavirin (Crotty et al., 2001; Contreras et al., 2002). The biological significance of these observations remains elusive.

Crotty and colleagues reported that, in particular, C-to-U (10-fold increase) and G-to-A (13.6-fold increase) mutations accumulate in the genome of poliovirus cultured in the presence of ribavirin. In our analysis, at most a 2.4-fold increase in C-to-U mutation frequency was observed in the ribavirin-treated virus. However, a similar 2.3-fold increase in C-to-U mutation frequency was observed in cultures treated with MPA, a compound that cannot be incorporated into viral RNA. No marked increase in G-to-A mutation frequency was observed for any of the treatment conditions. An increased mutation frequency observed in the genome of poliovirus grown for one passage in the presence of 1000 μM (224 μg/ml) ribavirin, which is comparable with the concentration used in the present study (i.e., 180 μg/ml or 738 μM), was thus not observed in the genome of YFV grown in the presence of ribavirin. Yet, the antiviral pressure on YFV may have been greater than in the poliovirus study, because the virus used for the mutation analysis had been passaged twice in the presence of high concentrations of ribavirin. Furthermore, in the present study, 1) complete extinction of viral replication was achieved, allowing analysis of the mutation frequency in pre-extinction YFV populations; and 2) mature YFV virus, which was released in the culture supernatant, was used for analysis of infectivity and mutation frequency in the genome, whereas poliovirus RNA was extracted from cytoplasmic lysates that may contain immature virus particles as well as replication complexes.

Day et al. (2005) recently reported extinction of West Nile virus replication when this virus was cultured in HeLa cells in the presence of 47 μg/ml ribavirin ($\approx$5-fold the EC_{90} for ribavirin against WNV in this cell line). However, a concentration as high as 100 μg/ml did not inhibit WNV replication in any of three monkey kidney cell lines tested. In our hands, a concentration of 100 μg/ml ribavirin did elicit a strong antiviral effect against YFV replication in African green monkey kidney cells, and, at a concentration of 120 μg/ml, it caused extinction of viral replication within five passages. The differences between the present study and the Day et al.

TABLE 3
Mutation frequency
Total number of mutations/1000 nt is shown. The x-fold difference with the untreated virus control is presented in parentheses.

Mutation	Virus Control	Ribavirin	EICAR	MPA
A to G	0.465	0.291 (0.6)	0.291 (0.6)	0.279 (0.6)
U to C	0.465	0.226 (0.5)	0.776 (1.7)	0.248 (0.5)
C to U	0.093	0.226 (2.4)	0.032 (0.3)	0.217 (2.3)
G to A	0.217	0.194 (0.9)	0.129 (0.6)	0.279 (1.3)

(2005) study may in part be explained by differences in cell culture system but maybe also by particular characteristics of each virus. Likewise, the replication of GB virus B, a virus closely related to hepatitis C virus, seems to be highly susceptible to error-prone replication induced by ribavirin in primary tamarin hepatocyte cell cultures (Lanford et al., 2001). Maag et al. (2001) reported that the RNA-dependent RNA polymerase of hepatitis C virus is able to incorporate ribavirin in an in vitro polymerization reaction. On average, 10^{-4} to 10^{-5} mutations per nucleotide are accepted per round of RNA replication of RNA viruses (Domingo et al., 1996; Drake et al., 1998). However, the error rate of the yellow fever virus RNA-dependent RNA polymerase was demonstrated to be as low as 1.9×10^{-7} to 2.3×10^{-7} (Pugachev et al., 2004). It remains to be studied whether differences in error rate between WNV (and other Flaviviridae) and YFV RNA-dependent RNA polymerases may explain the observations.

There is currently still no consensus whether ribavirin exerts its antiviral effect in patients with chronic HCV infection by induction of error-prone replication (Perelson and Ribeiro, 2005). No increase in mutation rate was detected in HCV genomes isolated from patients treated with ribavirin (Lutchman et al., 2004; Pawlotsky et al., 2004) or ribavirin/interferon combination therapy (Schinkel et al., 2003; Pawlotsky et al., 2005). In contrast, in a subset of ribavirin-treated, HCV-infected patients, a mutagenic effect of ribavirin could indeed be observed (Asahina et al., 2005). However, no conclusive evidence has been put forward so far.

In conclusion, our data provide compelling evidence that error-prone replication, leading to error catastrophe, is not involved in the in vitro antiviral effect of ribavirin (and other IMPDH inhibitors) against YFV replication. They also reinforce the conclusion reached from our previous study (Leyssen et al., 2005) that the predominant mechanism by which ribavirin inhibits YFV replication in cell culture is mediated by inhibition of IMPDH activity.

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