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Reversal of the antiviral activity of ribavirin against Sindbis virus in *Ae. albopictus* mosquito cells

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Summary

Earlier work in our laboratory has shown that the replication of Sindbis virus in *Aedes albopictus* mosquito cells is inhibited by ribavirin (Rbv) and mycophenolic acid (MPA) (Sarver and Stollar (1978) Virology 91, 267–282; Malinoski and Stollar (1980) Virology 102, 473–476). We report here that the antiviral effect of Rbv and MPA can be reversed by depriving infected cells of methionine or isoleucine, or by treating them with fluorodeoxyuridine (FUdR) or cycloleucine. We suggest that, as was the case when the antiviral activity of Rbv was reversed by actinomycin D (Malinoski and Stollar (1981a) Virology 110, 281–291), these effects may be mediated by changes in the GTP pools of treated cells.

Sindbis virus; *Aedes albopictus* mosquito cells; Ribavirin; GTP pools

Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Rbv) is a guanosine analog that is active in vitro against a wide range of RNA and DNA viruses (Sidwell, 1980). Clinically, it is used to treat patients infected with respiratory syncytial or Lassa fever virus (Hall et al., 1983; McCormick et al., 1986). In the cell, Rbv is converted to Rbv monophosphate, an inhibitor of inosine monophosphate dehydrogenase (IMPDH) (Streeter et al., 1973), the enzyme which catalyzes the first step specific for the de novo synthesis of GMP. Mycophenolic acid, although not a guanosine analog, is also a potent inhibitor

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of IMPDH (Smith et al., 1974). Both compounds, by inhibiting IMPDH, reduce the intracellular levels of GTP. Earlier work from this laboratory demonstrated that the inhibition of Sindbis virus (SV) replication in *Ae. albopictus* cells by Rbv or MPA correlated well with the reduction of cellular GTP levels (Malinoski and Stollar, 1981; Malinoski and Stollar, 1981b).

SV_{MPA} and SV_{LM21} are mutants isolated from standard SV (SV_{STD}) in our laboratory (Scheidel et al., 1987; Durbin and Stollar, 1985). SV_{MPA} is resistant to both MPA and Rbv, and SV_{LM21} is able to replicate in *Ae. albopictus* cells deprived of methionine. The causal mutations for both the SV_{LM21} phenotype and the SV_{MPA} phenotype have been mapped to the region of the genome encoding the nonstructural protein nsP1 (Mi et al., 1989; Scheidel and Stollar, 1991).

In the course of investigating whether SV_{LM21} and SV_{MPA} could complement each other, we found that the inhibition of SV_{LM21} replication in *Ae. albopictus* cells by Rbv (or MPA) was reversed by depriving the cells of methionine. This finding was reminiscent of an earlier report from our laboratory to the effect that actinomycin D both reversed the inhibition of Sindbis virus replication in *Ae. albopictus* cells by Rbv and restored the cellular pools of GTP to normal levels (Malinoski and Stollar, 1980; Malinoski and Stollar, 1981a). We describe here the reversal of the antiviral action of Rbv by methionine deprivation, and by several other conditions which inhibit cell growth.

Materials and Methods

Cells, media and viruses

The *Aedes albopictus* mosquito cells, clone C7-10, were cultured at 28° in E medium supplemented with 5% fetal calf serum as described previously (Durbin and Stollar, 1984). E medium is Eagle's minimal medium to which are added the nonessential amino acids and glutamine. The primary chick embryo cell cultures were prepared as described by Stollar et al. (1976). The viruses used in this study were our standard Sindbis virus SV_{STD} (Shenk and Stollar, 1973), SV_{MPA} which is resistant to mycophenolic acid and ribavirin (Scheidel et al., 1987), and SV_{LM21} which is able to replicate in methionine-deprived *Aedes albopictus* cells (Durbin and Stollar, 1985).

Virus assays

Virus replication was monitored both by plaque formation and by yield assays. Plaque formation was assayed on confluent *Ae. albopictus* monolayers as described by Durbin and Stollar (1984), except that the overlay medium contained 0.1% BSA instead of 5% serum. For the yield assays, virus was grown on *Ae. albopictus* cell monolayers and assayed by plaque formation on CEF monolayers (Shenk et al., 1974).

GTP Pools

Cultures of growing *Ae. albopictus* cells were labeled for 72 h with [^{32}P]inorganic phosphate and then treated as indicated. Acid-soluble extracts were then prepared and analysed by thin-layer chromatography (Stollar and Malinoski 1981b).

Chemicals

Cycloleucine and fluorodeoxyuridine were purchased from Sigma. Mycophenolic acid was the gift of Dr. K.F. Kosh, Lilly Research Laboratories. Ribavirin was purchased from ICN.

Results

As shown in Table 1, the replication of SV_{STD} in mosquito cells was, as expected, sensitive to both Rbv and methionine deprivation. The yield of virus from cells simultaneously deprived of methionine and treated with Rbv was similarly low.

SV_{MPA} was relatively resistant to Rbv but was sensitive to methionine deprivation. As with SV_{STD}, when SV_{MPA}-infected cells were deprived of methionine and at the same time treated with Rbv, little virus was produced.

With SV_{LM21} the situation was different. When the culture medium contained the normal level of methionine (100 μM), Rbv reduced the 18 h yield of SV_{LM21} from *Ae. albopictus* cells almost 4000-fold. However, when the ribavirin-treated cultures were also deprived of methionine, the yield was only about 50-fold less than that from the control culture (EA medium), or very similar to that obtained from methionine-deprived cells in the absence of ribavirin. Thus when cells were starved for methionine, ribavirin had no detectable effect on the replication of SV_{LM21}. As shown in Table 1, this effect could not be demonstrated with either SV_{STD} or SV_{MPA} because they are not resistant to methionine starvation.

TABLE 1

The effect of methionine deprivation on the inhibition of Sindbis virus replication by ribavirin

Virus	Yield of virus (pfu/ml)				Fold inhibition		
	EA(a)	Rbv(b)	M-O(c)	RbV:M-O(d)	a/b	a/c	a/d
SV_{STD}	8.9×10^9	2.9×10^6	2.1×10^6	1.3×10^6	3100	4200	6800
SV_{MPA}	2.1×10^9	4.7×10^7	1.7×10^6	5.5×10^5	45	1200	3800
SV_{LM21}	1.2×10^{10}	2.8×10^6	2.3×10^8	2.2×10^8	4300	52	55

Ae. albopictus cell monolayers were infected with SV_{STD}, SV_{MPA} or SV_{LM21} at an MOI of 10 pfu/cell as calculated from titers determined on mosquito cells. Medium was harvested at 18 h post-infection, and the titer of the infectious virus was assayed by plaque formation on monolayers of primary CEF as described in Materials and Methods. EA, E medium containing 0.1% BSA; Rbv, EA medium containing 500 μM ribavirin; M-O, EA medium lacking methionine; Rbv:M-O, EA medium lacking methionine and containing 500 μM ribavirin. All the fold inhibition values were calculated by comparing yields to the untreated controls, i.e., in EA medium.

TABLE 2

Plaquing efficiency of Sindbis virus on *Ae. albopictus* cells deprived of methionine and treated with Rbv or MPA

	EA	Rbv	MPA	M-O	Rbv:M-O	MPA:M-O
SV_{STD}	3.0×10^7	$<10^2$	$<10^2$	$<10^2$	$<10^2$	$<10^2$
SV_{MPA}	3.9×10^8	4.2×10^8	3.2×10^6	$<10^2$	$<10^2$	$<10^2$
SV_{LM21}	2.5×10^8	$<10^2$	$<10^2$	2.5×10^8	2.1×10^8	1.9×10^8

Plaque formation by SV_{STD}, SV_{MPA} and SV_{LM21} was performed on monolayers of *Ae. albopictus* cells as described in Materials and Methods. Plaques were counted 2 days after infection. MPA indicates EA medium containing 50 μM mycophenolic acid. MPA:M-O is M-O containing 50 μM mycophenolic acid. The other media are described in Table I.

The ability of methionine deprivation to reverse the effect of ribavirin was more striking when we measured the plaquing efficiency of our Sindbis virus stocks on *Ae. albopictus* cells (Table 2). When ribavirin or MPA was included in the overlay medium, the titer of SV_{LM21} was less than 10^2 pfu/ml compared to 2.5×10^8 pfu/ml in the absence of any drug. However, when the overlay medium contained no methionine, the plaquing efficiency was not affected by the presence of Rbv or MPA; in each case the titer of SV_{LM21} was the same as

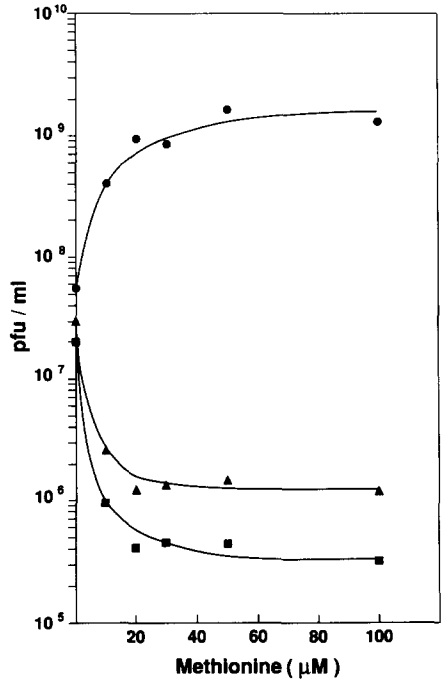

Fig. 1. *Ae. albopictus* cell monolayers were infected with SV_{LM21} at an MOI of 10 pfu/cell. After infection cells were maintained in EA medium modified to contain methionine at the indicated concentrations and MPA (50 μM) or Rbv (500 μM) as indicated. Medium was harvested 15 h after infection and assayed for infectious virus by plaque formation on *Ae. albopictus* cells. ▲, Rbv; ■, MPA; ●, neither Rbv nor MPA added.

TABLE 3

Reversal of the antiviral action of Rbv by FUdR, cycloleucine, or isoleucine deprivation

Modifications to the Medium				Yield of Virus pfu/ml	Fold Inhibition
FUdR	CL	I-O	Rbv		
–	–	–	–	3.0×10^{10}	–
–	–	–	+	2.6×10^{7}	1200
+	–	–	–	1.8×10^{10}	1.7
+	–	–	+	4.3×10^{9}	7
–	+	–	–	1.4×10^{10}	2.1
–	+	–	+	1.4×10^{9}	20
–	–	+	–	1.1×10^{9}	27
–	–	+	+	1.4×10^{9}	20

Ae. albopictus cell monolayers were infected with SV_{LM21} at an MOI of 10 pfu/cell, and then maintained in EA medium with additions or modifications as indicated. Medium was harvested 20 h later, and assayed for infectious virus by plaque formation on primary CEF cells. EA medium was described in Table I. FUdR indicates EA medium containing 1 μg FUdR/ml; CL indicates EA medium containing 5 mg cycloleucine/ml; I-O, EA medium lacking isoleucine; Rbv, EA medium containing 500 μM Rbv. All the fold inhibition values were calculated by comparing yields to the untreated control (no inhibitors at all).

in the control EA medium in the absence of any drug. As in the experiment shown in Table 1, the effect of methionine deprivation could only be shown with SV_{LM21}, and not with SV_{STD} or SV_{MPA}.

The effect of varying concentrations of methionine on the reversal of the antiviral action of MPA and Rbv is shown in Fig. 1. With a normal methionine concentration (100 μM) in the medium, Rbv and MPA reduced the yield of SV_{LM21} about 1000-fold and 3000-fold, respectively. Reducing the methionine concentration to 20 μM did not affect the antiviral activity of either of these compounds. However, when the concentration of methionine was further reduced to 10 μM, the inhibition by Rbv or MPA was only between 150- and 400-fold; and confirming the results shown in Table 1, in the absence of methionine, the yields from cultures treated with Rbv (or MPA) were not significantly different from cultures which received no Rbv (or MPA).

We next wished to know whether other conditions which inhibited cell growth would also reverse the antiviral effect of Rbv. Table 3 shows that in yield experiments, FUdR, an inhibitor of DNA synthesis (1 μg/ml), cycloleucine an inhibitor of *S*-adenosylmethionine synthetase (Caboche and Bachellerie, 1977) (5 mg/ml), and isoleucine deprivation all reversed the inhibition of SV_{LM21} replication by Rbv. Similar results were found with MPA (not shown). FUdR and cycloleucine alone had no effect on viral replication. Isoleucine deprivation alone caused only a 27-fold reduction in virus yield.

We also tested the influence of these conditions on the antiviral action of Rbv as assayed by plaquing efficiency. As indicated in Table 4, cycloleucine alone greatly reduced the size of the SV_{STD} plaques; and no plaques were detected even at low dilution when SV_{STD} infected-cells were overlaid with 500 μM Rbv plus cycloleucine. However, with SV_{LM21}, cycloleucine clearly did reverse the antiviral effect of Rbv.

TABLE 4

Plaquing efficiency of Sindbis virus on *Ae. albopictus* cells: Reversal of inhibition by Rbv

Medium	EA	Rbv	CL	FUdR	I-O	Rbv:CL	Rbv:FUdR	Rbv:I-O
Virus								
Experiment No. 1								
SV_{STD}	4.3×10^7	$<10^2$	$\approx 2 \times 10^{7(a)}$			$<10^{2(b)}$		
SV_{MPA}	4.4×10^8	5.9×10^8	2.3×10^8			$\approx 2 \times 10^{8(b)}$		
SV_{LM21}	2.9×10^8	$<10^2$	2.4×10^8			$2.4 \times 10^{8(b)}$		
Experiment No. 2								
SV_{STD}	4.2×10^7	$<10^2$		4.3×10^7	2.6×10^7		7.3×10^7	$\approx 10^{7(a)}$
SV_{MPA}	5.5×10^8	2.9×10^8		6.0×10^8	4.3×10^8		5.5×10^8	4.7×10^8
SV_{LM21}	4.7×10^8	$<10^2$		4.8×10^8	2.0×10^8		1.8×10^8	3.3×10^8

Stocks of SV_{STD}, SV_{MPA} and SV_{LM21} were titered under the indicated conditions by plaque formation on *Ae. albopictus* cells as described in Materials and Methods. The modifications of the media were described in Table 3. EA indicates EA medium with no modifications. [(a)]Plaques were very tiny and difficult to count accurately. [(b)]Plaque formation under these conditions was suboptimal, possibly due to toxic effects on the cells.

FUdR alone did not inhibit the plaque-forming ability of SV_{STD} on the *Ae. albopictus* cells; nevertheless, it did prevent the inhibitory effect of Rbv on both SV_{STD} and SV_{LM21}. Similarly, isoleucine deprivation had no effect on plaque formation by SV_{STD}, SV_{MPA} or SV_{LM21} but was able to reverse the inhibition by Rbv of SV_{STD} and SV_{LM21} (the plaques formed under these conditions by SV_{STD} were, however, tiny).

As already noted, when actinomycin D reversed the antiviral action of Rbv, it also restored the cellular pool of GTP to a normal level. We therefore wished to know whether the same would be true of the various treatments we describe in this paper which reverse the antiviral action of MPA and Rbv.

As expected, Rbv reduced the level of GTP in *Ae. albopictus* cells (Fig. 2A, lane B vs. lane A). Addition of FUdR, or deprivation of methionine or isoleucine on the contrary led to a small increase in the GTP level (lanes C, D, and E). When cells were treated with both FUdR and Rbv (lane F) the GTP level was similar to that in the control cultures (lane A), and significantly more than in the culture treated with Rbv alone (lane B). When Rbv-treated cells (lanes G and H) were deprived of methionine or isoleucine (lane G and H), the GTP levels were somewhat increased over that in the cultures treated only with Rbv (lane B), but the reversal was not as dramatic in this experiment as with FUdR. However, as shown in Fig. 2B, methionine-deprivation did dramatically reverse the reduction in GTP brought about by MPA (lanes D vs. lane B). Methionine-deprivation alone (lane C) had no effect on the GTP level.

These results are consistent with the hypothesis that drugs or conditions which reverse the antiviral action of Rbv or MPA, do so by their effects on intracellular levels of GTP. The mechanisms involved, however, may be quite complex.

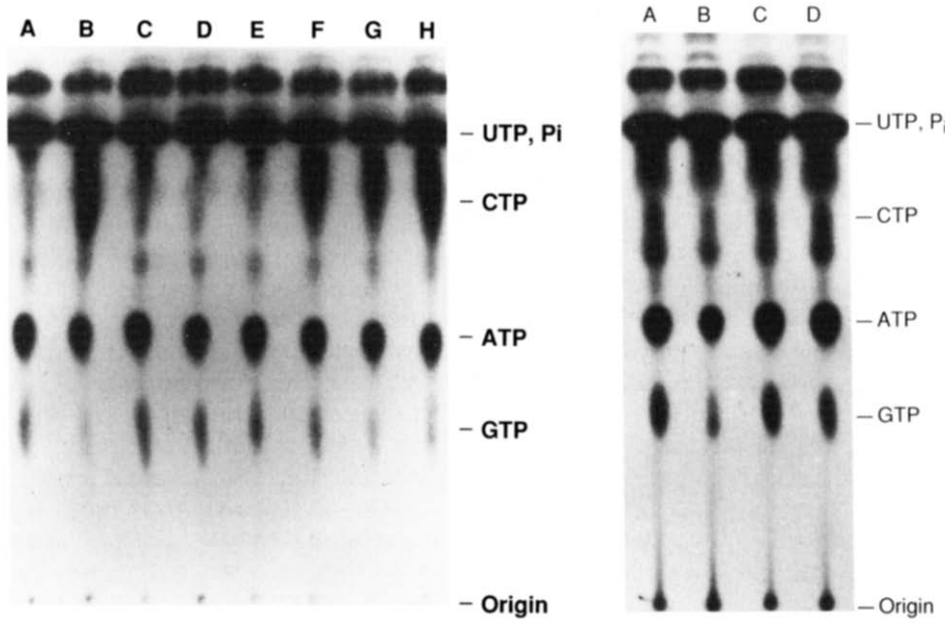

Fig. 2. GTP levels in *Ae. albopictus* cells. Effects of Rbv and MPA and conditions which reverse the antiviral effects of these compounds. A. Growing cultures of *Ae. albopictus* cells were labeled with [^{32}P]inorganic phosphate for 3 days and then collected by centrifugation. Equal numbers of cells suspended in fresh EA medium modified as indicated below, and containing as before [^{32}P]inorganic phosphate, were distributed to test tubes. Cells were then harvested 4 1/2 h later. A, no additions or modifications. B, 500 μM Rbv. C, 1 μg FUdR/ml. D, M-O medium. E, I-O medium. F, 500 μM Rbv plus 1 μg FUdR/ml. G, 500 μM Rbv in M-O medium. H, 500 μM Rbv in I-O medium. B. Cells in 60 mm plates were labeled with [^{32}P]inorganic phosphate for 3 days. The medium was then replaced with fresh EA or M-O medium, again containing [^{32}P]inorganic phosphate, and 50 μM MPA as indicated. Cells were harvested 15 h later. A, EA medium. B, EA medium plus MPA. C, M-O medium. D, M-O medium plus MPA.

Discussion

Three mechanisms have been suggested as explanations for the antiviral effect of Rbv. (i) inhibition of IMPDH and depletion of the cellular GTP pool (Streeter et al., 1973). In support of this mechanism is the correlation between the antiviral activity of Rbv against Sindbis virus and the levels of GTP in the infected cells (Malinoski and Stollar, 1981a; Malinoski and Stollar, 1981b). (ii) inhibition of viral RNA polymerase by Rbv triphosphate. Influenza virus, vesicular stomatitis virus, and La Crosse virus RNA polymerases are all inhibited by Rbv triphosphate in vitro (Wray et al., 1985; Toltzis et al., 1988; Cassidy and Patterson, 1989). In the case of vesicular stomatitis virus and La Crosse virus polymerases there is evidence that the block in replication occurs at the level of initiation of transcription (Fernandez-Larsson et al., 1989; Cassidy and Patterson, 1989). In vitro transcription of mRNA by reovirus

cores was also inhibited by Rbv triphosphate (Rankin et al., 1989). (iii) inhibition of the RNA 5′ guanylyltransferase capping enzyme by Rbv triphosphate (Goswami et al., 1979). The only case in which this has been demonstrated is for vaccinia virus. Cap formation in vitro by reovirus cores was not sensitive to Rbv triphosphate (Rankin et al., 1989).

In earlier work we observed a strong correlation between inhibition of viral replication by Rbv and depletion of the GTP pool (Malinoski and Stollar, 1981a; Malinoski and Stollar, 1981b). When Rbv inhibited virus replication, the GTP levels were low; in cells in which Rbv did not inhibit viral replication or when treatment with actinomycin D abolished the antiviral action of Rbv, the GTP levels remained close to normal.

It is likely that the reversal of the antiviral action of Rbv and MPA, described here, is also mediated through effects on the GTP pool. Since GTP can be considered a substrate for two virus-coded enzymes, the RNA-dependent RNA polymerase and the RNA guanylyltransferase (capping enzyme), the depletion of GTP by Rbv or MPA could affect primarily viral RNA synthesis, RNA capping, or possibly both. Since, however, the causal mutations for resistance of Sindbis virus to MPA and Rbv map to the region of the genome which encodes nsP1 (Scheidel and Stollar, 1991), and not to the region which encodes nsP4, the viral RNA-dependent RNA polymerase, we conclude that the primary effect of the lowered GTP pool is to block the capping of the viral RNA. This does not rule out the possibility that, as reported for vaccinia virus, the Sindbis virus capping enzyme is also directly inhibited by the triphosphate form of Rbv. However, there is at present no evidence to support this idea.

Our findings demonstrate that several different treatments can abolish the antiviral effect of Rbv in mosquito cells. These different treatments have in common the property of inhibiting cellular growth. On the basis of these results, it would not be surprising if, in whole organisms, the antiviral effect of Rbv might be expressed to varying degrees in different tissues or organs. In vitro we have shown that Rbv consistently inhibits the replication of SV in mosquito cells (Sarver and Stollar, 1978; Malinoski and Stollar, 1981a) but not in chick cells (Malinoski and Stollar, 1981b). In BHK cells the antiviral activity of Rbv is influenced by several factors including the growth state of the cells (Malinoski and Stollar, 1981b). The demonstration that the effect of Rbv on viral replication is host cell dependent may have implications related to the effectiveness of Rbv for treating viral infections of different organ systems.

It bears emphasis that our initial observation, namely that methionine deprivation reverses the antiviral effect of Rbv and MPA, was made possible only by virtue of our having the SV_{LM21} mutant, which is capable of replication in methionine-starved cells.

Most antiviral drugs, or their active forms, acyclovir and AZT for example, interact with a virus-coded protein (Elion, 1982; Furman et al., 1986). In the case of Rbv, the weight of evidence indicates that its antiviral activity derives from the inhibition not of a viral enzyme, but a cellular enzyme, IMPDH. This

may explain the relatively broad antiviral spectrum of Rbv (at least in cultured cells), relative to that of AZT and acyclovir. However, a better understanding of how Rbv inhibits viral replication will likely require the study of viral RNA synthesis in cell-free systems with purified components.

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