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INHIBITION OF SINDBIS VIRUS REPLICATION BY RIBAVIRIN: INFLUENCE OF CULTURAL CONDITIONS AND OF THE HOST CELL PHENOTYPE

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Several factors which influence the ability of ribavirin to inhibit the replication of Sindbis virus in baby hamster kidney (BHK) cells are described; these include the cell passage level, the presence of serum in the medium and cell density. Inhibition of viral replication was much more effective with low passage cells than with high passage cells. However, with high passage cells antiviral activity was enhanced 1) when subconfluent cultures were infected, rather than confluent cultures, and 2) when infected cultures were maintained in the presence of serum rather than without serum. Under all conditions, a good correlation was found between the inhibition of virus replication by ribavirin and its ability to reduce cellular GTP levels. Using Chinese hamster ovary cell mutants we found that ribavirin inhibited Sindbis virus replication in cells lacking hypoxanthine phosphoribosyltransferase ($HPRT^-$) but not in $HPRT^+$ cells. This result and the demonstration that *Aedes albopictus* cells (in which ribavirin is very effective) are unable to incorporate hypoxanthine suggest that certain salvage enzymes involved in purine metabolism can interfere with the antiviral activity of ribavirin.

ribavirin Sindbis virus

INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole carboxamide; Rbv) is a nucleoside analog which inhibits the replication of a wide range of DNA and RNA viruses [19]. At least three mechanisms of action have been proposed to account for this antiviral activity: a) inhibition by Rbv monophosphate of inosine monophosphate dehydrogenase (IMPDH), the first enzyme specific for the synthesis of guanosine monophosphate (GMP), leading in turn to a reduction in GTP available for viral nucleic acid synthesis [23], b) inhibition of virion RNA polymerase by Rbv triphosphate [4], or c) inhibition of RNA capping enzymes by Rbv triphosphate [7].

In studies of the antiviral activity of Rbv and other IMPDH inhibitors in *Aedes albopictus* cells, we found that the inhibition of Sindbis virus replication by these compounds correlated very well with a reduction of cellular GTP levels [12]. In addition, conditions which reversed the antiviral activity of these inhibitors, e.g. treatment with actinomycin D, also restored GTP levels to near normal.

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However, when we tested the effect of Rbv in baby hamster kidney (BHK) cells the results were variable; in some experiments there was no inhibition and in others some inhibition was observed. Our findings with BHK cells differed therefore from those in other reports in which Rbv inhibited the replication of mumps, Sindbis, and St. Louis encephalitis viruses in BHK cells (reviewed in ref. 18) and from our results with *A. albopictus* cells in which Rbv consistently inhibited the replication of Sindbis virus. We therefore set out to systematically investigate the factors which influence the inhibition by Rbv of virus replication in BHK cells. We found that the passage level of the cells, the presence of serum in the medium and the cell density were critical factors in determining whether Rbv was able to inhibit virus replication. In these experiments, as in our earlier ones [12], there was a good correlation between inhibition of virus replication and a reduction in GTP levels. The possible role of purine salvage enzymes in influencing the effectiveness of Rbv was also examined by comparing the activity of Rbv in cells lacking hypoxanthine phosphoribosyltransferase ($HPRT^-$) and in $HPRT^+$ cells.

MATERIALS AND METHODS

Cells, media and virus

Baby hamster kidney cells (BHK-21) from the American Type Culture Collection were grown at 37°C in monolayer culture and then after one to two passages frozen in ampoules and stored in the vapor phase of a liquid nitrogen freezer. They were routinely passaged three times a week for three to four months (approximately 40 passages). After this, a new ampoule of cells was thawed to begin another passage series. The human KB cell line and African green monkey kidney cells (Vero) were also obtained from the American Type Culture Collection. The preparation of primary chick embryo fibroblast (CEF) cultures and the *Aedes albopictus* cells (clone LT C-7) have been described elsewhere [10, 16]. The Chinese hamster ovary (CHO) cell lines 2F3 and EOH-3 were obtained from Dr. L. Siminovitch (Toronto, Canada). The 2F3 cells are thymidine kinase-deficient (TK^-) and require proline for growth. The EOH-3 cells also require proline, are resistant to emetine and ouabain, and lack hypoxanthine phosphoribosyl transferase (HPRT) activity. Cells were counted with a Coulter Counter, Model ZBI.

HT medium [14] was used for growing BHK cells. KB and Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 2 mM glutamine, antibiotics, and 10% heat-inactivated (56°C, 30 min) calf serum. CHO cells were maintained in monolayer culture at 34.5°C in complete α-MEM [20] supplemented with antibiotics, 2 mM glutamine, 1 mM Hepes buffer, and 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS). *A. albopictus* cells, clone LT C-7, were maintained at 28°C in E medium [16] (MEM containing antibiotics, 2 mM glutamine, 2 × MEM non-essential amino acids) supplemented with 10% FCS; CEF cells were grown at 37°C as described elsewhere [10].

The stocks of Sindbis virus (SV) were prepared as described previously [22]. Cells

were infected with an input multiplicity of approximately 10 plaque-forming units (p.f.u.) per cell. Adsorption was at 34.5°C for 45 min, after which monolayers were washed with phosphate-buffered saline (PBS). After infection all cell types were maintained in E medium containing either 10% FCS (E-10) or no serum (E-0) and then were incubated at 34.5°C. Yields of infectious virus were measured by plaque assay on monolayers of CEF [17].

Measurement of intracellular nucleotide pools

Two methods were used to examine the effects of Rbv on the nucleotide pools. In the first, growing cells were labeled for 90 h with ortho[^{32}P]phosphate (5 μCi/ml). The cultures were then treated with Rbv as indicated after which acid-soluble extracts were prepared [8] and the ribonucleoside triphosphates analyzed by thin-layer chromatography [3] on polyethyleneimine-cellulose thin layer plates (PEI-cellulose; Brinkman).

In the second method, acid-soluble extracts of unlabeled cells were prepared as above and analyzed by high performance liquid chromatography (HPLC) essentially as described by Brown [2]. A Waters Associates HPLC was used which was equipped with a Model 6000 A pump, a Model 45 pump and a Model 660 Solvent Programmer. A Partisil 10 SAX anion-exchange column (4.6 mm × 25 cm; Whatman, Inc.) was used and samples were injected with a Model U6K injector system (Waters Assoc.). Samples were eluted from the column at a flow rate of 1.5 ml/min using a linear gradient from 0.01 M $NH_4H_2PO_4$ (pH 3.2) to 0.25 molal $NH_4H_2PO_4$ in 0.5 molal KCl (pH 4.2). The gradient was run for 60 min after which elution of the samples was completed by pumping under final conditions for an additional 20 min. The absorbance of eluted material was determined at 254 nm by a Waters Assoc. Model 440 UV-Detector and peaks were quantitated by measuring the areas under the curves. Concentrations were determined using calibration curves for each of the nucleotides of interest.

Incorporation of purine bases

Confluent cell cultures were refed with E-10 medium containing 5 μCi of [^{3}H]adenine or [^{3}H]hypoxanthine per ml of medium and incubated at the indicated temperature. 90 min later, monolayers were washed three times with ice-cold PBS after which acid-insoluble material was precipitated with 0.4 M perchloric acid (PCA) for 15 min on ice. Acid-insoluble material was then washed with ethanol (95%) and methanol to remove PCA and then solubilized with 1 M NaOH at 65°C for 15 min. A portion of this material was then neutralized with acid and counted in Formula 963 (New England Nuclear) to determine incorporation into acid-insoluble material.

Chemicals and isotypes

Ribavirin, [^{3}H]adenine (16.6 Ci/mmol), [^{3}H]hypoxanthine (3.6 Ci/mmol), and

$^{32}PO_4$ (carrier-free) were all purchased from ICN. HPLC-grade $NH_4H_2PO_4$ was purchased from J.T. Baker and all other chemicals were obtained from Fisher Scientific or Sigma Chemical Corporation.

RESULTS

In our first experiments, when we treated BHK cells with Rbv, we did not observe any reduction in the yield of SV. Since these experiments were done with high-passage BHK cells (passage level greater than 20), we repeated them using BHK cells at a low passage level. This time there was an inhibition of virus replication of approximately 1000-fold. We therefore examined in greater detail the effect of Rbv on viral replication in BHK cells at various passage levels. In each case cultures were used just as they reached confluency. The results of one such experiment are presented in Table 1. At early passage levels, such as passage 3, Rbv was very effective in reducing virus yields (4000-fold reduction), whereas with cells at late passage (e.g. passage 23) Rbv produced little or no inhibition (< 2-fold).

Since the low passage BHK cells are more contact inhibited and attain lower saturation densities than higher passage level cells, we wished to know whether the antiviral activity of Rbv in high passage level cells might be influenced by factors such as cell density and rate of growth. Thus, we tested the antiviral activity of Rbv on high passage BHK cells which were confluent and on cells which had not reached confluency; and we tested the effect of Rbv on cultures maintained in either the absence or the presence of serum. Little or no inhibition (4-fold at 8 h; none at 24 h) of SV by Rbv was found with confluent cultures maintained after infection in serum-free medium (Table 2). In contrast, in subconfluent cultures refed with medium containing 10% FCS, Rbv reduced virus titers by 180-fold at 8 h and 9-fold at 24 h. When subconfluent cultures were refed with serum-free medium or confluent cultures were refed with serum-containing medium there was an intermediate inhibition of SV by Rbv (36-fold and 13-fold, respectively, at 8 h). In all cases, the degree of inhibition by Rbv was greater at 8 h than at 24 h. Fig. 1 illustrates graphically the results of a similar experiment, comparing the effects of Rbv on confluent and subconfluent cultures of BHK cells (both maintained in 10% FCS). These results confirm those presented in Table 2, but in addition illustrate that even when there is good inhibition at early times, as with subconfluent cultures (Panel A), by later times (24 h) virus yields from Rbv-treated cultures tended to approach the levels seen in untreated cultures.

Table 3 shows the levels of GTP (as measured by high performance liquid chromatography) under the different experimental conditions. Under conditions where Rbv was most effective in inhibiting virus replication (low cell density, high serum) there was, by 8 h after treatment, a 10-fold reduction in the amount of GTP. By 24 h, by which time the level of virus in the Rbv-treated cultures had increased (Table 2), the level of GTP had also risen very significantly. Under conditions in which virus replication was not inhibited by Rbv (high cell density, no serum) there was no significant reduction

TABLE 1

The effect of passage level on the inhibition of Sindbis virus replication by ribavirin in confluent cultures of BHK-21 cells

No. of cells/plate at time of infection ($\times 10^6$)	Passage level	Time (h after infection)	Yield of SV (p.f.u./ml)		Fold inhibition
			without RBV	with Rbv	
8.4	3	16	4.1×10^9	1.0×10^6	4000
8.2	7	16	4.2×10^9	1.1×10^7	380
6.9	13	16	5.5×10^9	3.4×10^8	16
7.7	15	16	8.0×10^9	2.0×10^9	4
9.2	23	16	1.7×10^{10}	8.0×10^9	2
9.2	23	24	1.2×10^{10}	1.0×10^{10}	< 2

BHK cells at different passage levels were seeded into 60 mm tissue culture plates so as to give confluent monolayers after growth at 37°C for 2 days. Cultures were then infected with SV and virus titers were assayed as described in Materials and Methods. The level of virus at 2 h after infection, a measure of desorbed virus, was $1-3 \times 10^6$ p.f.u./ml. Rbv was added after adsorption to give a final concentration of 6.4×10^{-4} M (150 μg/ml).

TABLE 2

The effect of cell density and serum on the inhibition of Sindbis virus replication by ribavirin in late passage BHK-21 cells

Serum concentration	Time (h after infection)	Yield of SV (p.f.u./ml) from subconfluent cultures			Yield of SV (p.f.u./ml) from confluent cultures		
		without Rbv	with Rbv	fold inhibition	without Rbv	with Rbv	fold inhibition
10%	8	2.2×10^9	1.2×10^7	183	7.0×10^9	5.3×10^8	13
	24	3.4×10^9	3.7×10^8	9	1.0×10^{10}	9.7×10^9	None
None	8	7.3×10^8	2.0×10^7	36	6.5×10^8	1.5×10^8	4
	24	2.1×10^9	3.5×10^9	None	5.1×10^9	5.5×10^9	None

Cells were infected with SV and virus titers were assayed as described in Materials and Methods. Subconfluent and confluent cultures contained 3.8×10^6 cells and 8.5×10^6 cells, respectively, per 60 mm plate at the time of infection. The level of virus at 2 h after infection was $1-3 \times 10^6$ p.f.u./ml and $2-5 \times 10^5$ p.f.u./ml in cultures refed with 10% FCS and 0% FCS, respectively. Rbv was added after virus adsorption to give a final concentration of 6.4×10^{-4} M.

TABLE 3

GTP levels (nmol/10^7 cells) in Sindbis virus-infected BHK-21 cells treated with ribavirin

Serum concentration	Time (h after infection)	GTP (nmol/10^7 cells) in subconfluent cultures			Fold inhibition of SV*	GTP (nmol/10^7 cells) in confluent cultures			Fold inhibition of SV*
		without Rbv (a)	with Rbv (b)	(b/a) × 100		without Rbv (a)	with Rbv (b)	(b/a) × 100	
10%	8	4.0	0.4	10	183	4.8	1.4	30	13
	24	7.0	3.0	43	9	6.7	4.6	69	None
None	8	4.2	0.8	19	36	4.9	4.4	90	4
	24	6.1	6.6	100	None	5.6	5.4	96	None

The levels of GTP in acid-soluble extracts of the cell monolayers used in the experiment presented in Table 2 were determined by HPLC as described in Materials and Methods. The concentration of Rbv was 6.4×10^{-4} M.

* From Table 2.

TABLE 4

The antiviral activity of ribavirin in various vertebrate cells

No. of cells/plate at time of infection (× 10^6)	Cell type	Time (h after infection)	Yield of SV (p.f.u./ml)		Fold inhibition
			without Rbv	with Rbv	
8.0	Chick embryo	16	2.4×10^9	1.6×10^9	< 2
6.8	Human KB	16	7.2×10^8	1.1×10^8	6.5
6.4	Monkey kidney (Vero)	16	1.2×10^9	9.1×10^7	13
		24	2.3×10^9	3.0×10^9	None

Cells were infected with SV and refed with medium E-10; virus yields were assayed as described in Materials and Methods. The level of virus at 2 h after infection ranged from 2 to 6×10^6 p.f.u./ml. Rbv was added after adsorption to give a final concentration of 6.4×10^{-4} M.

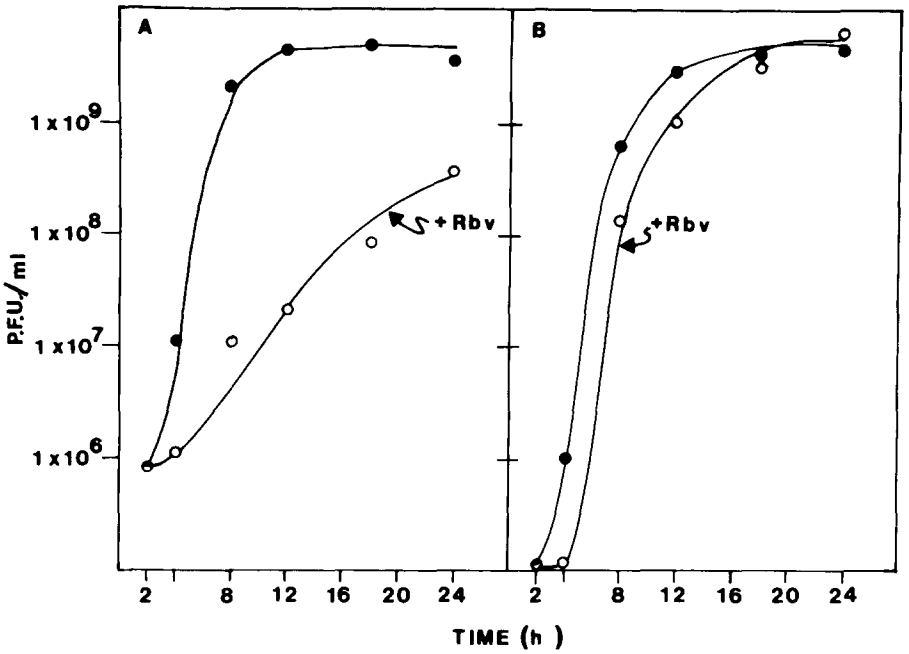

Fig. 1. The effect of Rbv on the replication of SV in subconfluent and confluent cultures of BHK-21 cells. Cells were infected with SV and virus yields assayed as described in Materials and Methods. After adsorption, subconfluent (A) and confluent (B) cultures were refed with E-10 medium either with (○) or without (●) Rbv (6.4×10^{-4} M). Subconfluent and confluent cultures contained 3.5×10^5 and 8.7×10^6 cells, respectively, per 60 mm plate at the time of infection.

in the concentration of GTP. Thus, there was an extremely good correlation between the effect of Rbv on virus yield and a reduction in the cellular GTP level.

The poor response to Rbv by confluent late passage cell cultures could be explained if Rbv were not taken up by these cells. We were able to test this possibility, since Rbv triphosphate is readily detected in acid-soluble extracts of $^{32}PO_4$-labeled Rbv-treated cells [12]. Fig. 2 shows an autoradiogram of $^{32}PO_4$-labeled acid-soluble extracts (analyzed by thin-layer chromatography) from late passage and early passage confluent cells with or without Rbv treatment. Rbv triphosphate (indicated by the arrow), which migrates just ahead of CTP, was present in extracts of both early and late passage cells. Thus Rbv must have been taken up by the late passage cells as well as by the early passage cells. The GTP level, however, was reduced only in the early passage cells.

In experiments with other vertebrate cell lines, Rbv showed a range of effectiveness. As seen in Table 4, Rbv did not inhibit SV replication in primary chick embryo cells but did to some extent in Vero (13-fold reduction in virus yield) and human KB cells (6-fold reduction). In these experiments cultures were infected after the cells had reached confluency.

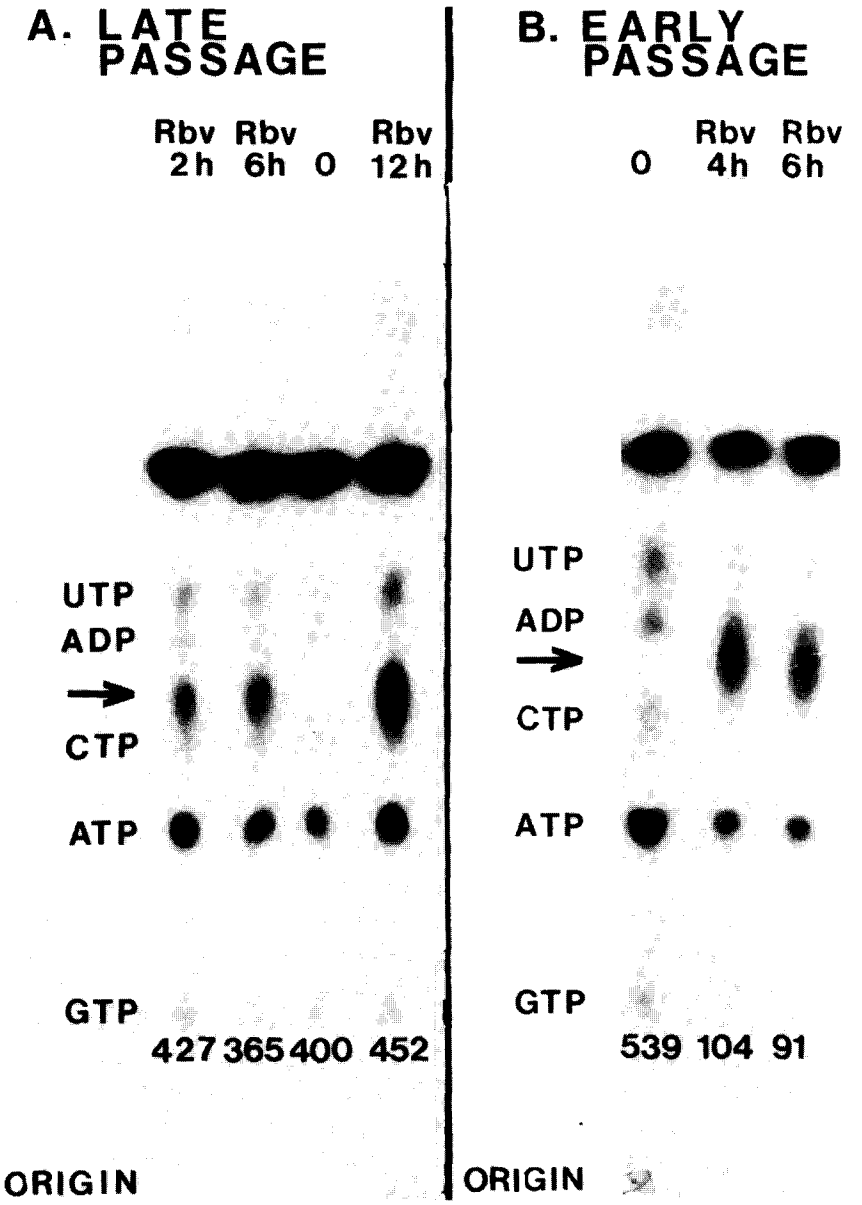

Fig. 2. Thin-layer chromatography of acid-soluble extracts of late and early passage BHK-21 cells treated with Rbv. Cells in medium containing 10% serum were labeled with $^{32}PO_4$ for 90 h as described in Materials and Methods. At zero time Rbv (6.4×10^{-4} M) was added to the cultures and acid-soluble extracts were prepared at the times indicated. The numbers below the GTP spots indicate the counts per minute in each spot for the various samples.

The inhibition of IMPDH by Rbv monophosphate prevents the conversion of IMP to xanthosine monophosphate (XMP), an intermediate in the synthesis of GMP (ref. 23, and see Fig. 3). This might be expected to result in an accumulation of IMP which could be converted either to adenosine monophosphate (AMP) by the other branch of the purine biosynthetic pathway or to other purine compounds by salvage enzymes (see Fig. 3). In low-density serum-fed BHK cells treated with Rbv, at late times after infection, GTP levels increased as virus titers increased (Table 3). This suggested that the effectiveness of Rbv might be influenced by the ability of these cells to use salvage enzymes to circumvent the Rbv-induced block in the de novo synthesis of GMP.

Since earlier work from this laboratory had indicated that *A. albopictus* cells do not incorporate hypoxanthine into nucleic acids (Mento, Sherwood and Stollar, unpublished observations), presumably because they lack HPRT, we thought that this might explain why Rbv is so effective in these cells. If this idea were valid, then Rbv should inhibit virus replication much better in mammalian cells which were $HPRT^-$ than in cells which were $HPRT^+$. As shown (Table 5) both *A. albopictus* and $HPRT^-$ CHO cells incorporated hypoxanthine into an acid-insoluble form very poorly, compared to their ability to incorporate adenine, another purine, In contrast, CHO cells which were $HPRT^+$ incorporated hypoxanthine quite well.

As shown in Table 6, there was indeed a difference in the effectiveness of Rbv in $HPRT^+$ and $HPRT^-$ CHO cells. In the absence of Rbv both cell types made similar amounts of Sindbis virus. In contrast, in the presence of Rbv, the yields of SV were reduced 50–100-fold in the $HPRT^-$ cells but not at all in the $HPRT^+$ cells.

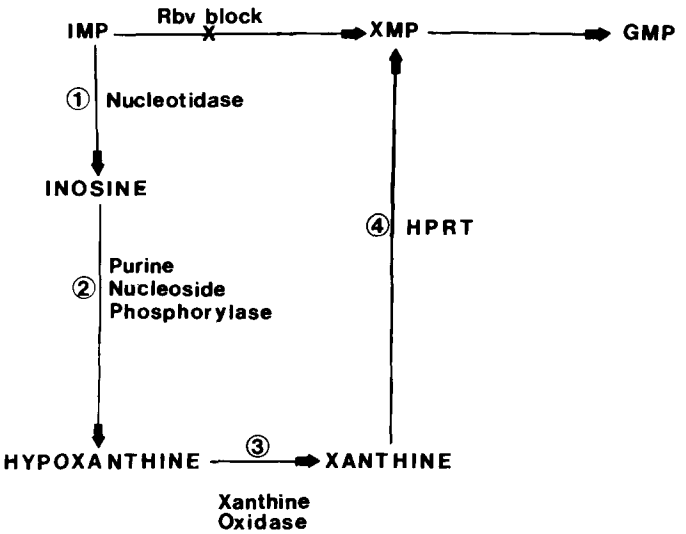

Fig. 3. A pathway by which cells might circumvent the block in de novo GMP synthesis caused by Rbv.

TABLE 5

Incorporation of [^{3}H]hypoxanthine and [^{3}H]adenine into acid-insoluble material by *A. albopictus* and Chinese hamster ovary cell lines

Cell line	[^{3}H]Hypoxanthine (c.p.m./10^6 cells)	[^{3}H]Adenine (c.p.m./10^6 cells)
A. albopictus (LT C7)	125	188,493
CHO EOH-3 ($HPRT^-$)	1,221	63,758
CHO 2F3 ($HPRT^+$)	16, 186	49,071

Monolayer cultures of cells received 5 Ci of [^{3}H]hypoxanthine (3.6 Ci/mmol) or [^{3}H]adenine (16.6 Ci/mmol) per ml of medium. After 90 min at 28°C (*A. albopictus* cells) or 34.5°C (CHO cells) incorporation into acid-soluble material was measured as described in Materials and Methods. Values represent the average of measurements done on triplicate cultures.

DISCUSSION

In both animal studies and clinical trials Rbv has been shown to have beneficial effects in the treatment of certain virus infections (e.g. measles, hepatitis, herpes zoster) but not others (e.g. influenza A, Chikungunya) [1, 5, 13, 21]. As noted in the review by Sidwell [18], in experiments with cultured cells all of the above viruses were reported to be inhibited by Rbv; however, in an earlier report it was demonstrated that the effectiveness of Rbv was also markedly influenced by the host cell [9]. This was shown especially clearly with herpes simplex, vesicular stomatitis, and vaccinia viruses which were poorly inhibited by Rbv in Vero cells, but were inhibited efficiently in other cell types. The data presented in this report describe some variables that can affect the antiviral activity of Rbv in an established cell line.

Our results indicate that with BHK cells, cell passage level, serum concentration, and cell density markedly influence the ability of Rbv to inhibit the replication of Sindbis virus. Such factors may account for the reported variability between cell lines with respect to the effectiveness of Rbv [9, 21].

We reported earlier [12] that in *A. albopictus* cells the inhibition of virus replication by Rbv correlated extremely well with a reduction in cellular GTP levels. In this report (Table 3) we noted that in those experiments in which virus replication was inhibited by Rbv at early times (8 h) the level of GTP was also reduced, but that at later times as the virus titers increased there was also an increase in the GTP levels. These observations taken together support the idea that the antiviral activity of Rbv is due mainly to an inhibition of the synthesis of GMP in the infected host cell [23].

We also found (see Fig. 2) that the presence of Rbv triphosphate in Rbv-treated cells did not always correlate with either an inhibition of virus replication or a reduction in the GTP level. This suggests that, at least for Sindbis virus, the reported effects of Rbv tri-

TABLE 6

Antiviral activity of Rbv in $HPRT^+$ and $HPRT^-$ Chinese hamster ovary cells

Time (h after infection)	Rbv (6.4×10^{-4} M)	Yield of SV (p.f.u./ml) from $HPRT^+$ cells (2F3)	Fold inhibition	Yield of SV (p.f.u./ml) from $HPRT^-$ cells (EOH-3)	Fold inhibition
8	-	1.3×10^9		7.0×10^8	
	+	7.0×10^8	< 2	6.0×10^6	116
24	-	2.5×10^9		4.9×10^9	
	+	3.1×10^9	None	9.3×10^7	53

Cells (4.5×10^6/60 mm plate) were infected with SV and the yields of virus were assayed as described in Materials and Methods. The level of virus at 2 h after infection was between 2 and 5×10^6 p.f.u./ml.

phosphate on viral polymerase [4] and mRNA capping [7] activities may not be crucial to the inhibition of virus replication by Rbv.

Our results also suggest that the activity of salvage enzymes, specifically HPRT, may interfere with the ability of Rbv to maintain a reduced level of GTP and thus with its antiviral activity. This could occur in two ways. First, it has been shown that the ribose phosphate of IMP can be transferred to guanine by HPRT, converting IMP and guanine to hypoxanthine and GMP, respectively [6]. Thus, if there were sufficient guanine available the block in de novo GMP synthesis caused by Rbv could be overcome. Secondly, as shown in Fig. 3, IMP can be converted to XMP by the sequence of reactions labeled 1, 2, 3 and 4, circumventing the block in de novo GMP synthesis. The first two reactions convert the nucleotide IMP to the free base hypoxanthine [24]. Hypoxanthine can then be converted to xanthine which can serve as a substrate for HPRT [11].

It is also possible that HPRT activity might vary with cell density and serum levels and that such changes in the activity of this enzyme might partially explain the variation we found in the effectiveness of Rbv in BHK cells. Furthermore, there are differences between normal and tumor cells and among different tissues with respect to the degree to which they rely on salvage versus de novo pathways to synthesize nucleotides [15]. Thus it would not be surprising if the sensitivity of virus replication to inhibitors of de novo purine synthesis like Rbv might vary in different cells, tissues or organs in vivo.

Somatic cell hybrids and gene transfer experiments could be used to learn more about the role of HPRT in influencing the effectiveness of Rbv. Such information would be important in the development of new antiviral agents or combination chemotherapy to treat viral infections refractory to treatment with ribavirin.

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