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BOVINE LEUKEMIA VIRUS INHIBITION IN VITRO BY RIBAVIRIN

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Ribavirin treatment of an established monolayer of fetal lamb kidney (FLK) cells chronically infected with bovine leukemia virus (BLV) resulted in inhibition of the extra- and intracellular internal viral polypeptide antigen, as measured by CF test, at concentrations of 10 and 32 μg/ml, respectively. Similar treatment of F-81 cells newly infected with BLV caused significant reduction in viral syncytia formation at ribavirin levels as low as 3.2 μg/ml. Attempts to eliminate or reduce the BLV infection in FLK cells by 8 passages of the cells in the continual presence of 3.2 or 1.0 μg/ml of ribavirin were unsuccessful. Multiple passages of FLK cells in the presence of higher concentrations of ribavirin substantially retarded cell growth, although short-term treatment of established cell monolayers appeared to be well tolerated as evidenced by cell appearance. Biochemical cytostatic studies of resting F-81 cell monolayers showed cytostatic effects at the same dosage levels where antiviral effects occurred.

ribavirin virazole 1-β-D-ribofurasonyl-1,2,4-triazole-3-carboxamide bovine leukemia virus

INTRODUCTION

The bovine leukemia virus (BLV), an oncogenic retrovirus, is widely distributed in cattle populations and is considered the etiologic agent of bovine lymphosarcoma or leucosis [16]. The disease induced by BLV appears to be increasing in economic importance as awareness of the disease heightens and additional requirements mount for testing for presence of BLV infection prior to export. The virus also has oncogenic potential in experimentally inoculated sheep [8, 15, 17] and goats [5].

Little has yet been accomplished to control BLV infections; a brief report [1] has described the use of an unidentified potential antiviral agent ('P-2', Polonine Development Lab., Woodside, NY) in treating BLV-positive cattle. The results of the trial were inconclusive. A recent report by Sundquist and Oberg [13] indicates that the antiviral, phosphonoformate, specifically inhibits bovine leukemia and related viral RNA polymerases. Several compounds are known to have a significant inhibitory effect on the development of C-type retroviruses and on the diseases induced by these viruses in laboratory animals. Among the more effective of these antiviral agents is 1-β-D-ribofuranosyl-1,2,4-triazole-

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3-carboxamide (ribavirin), a broad spectrum virus-inhibitory compound which is active in vitro against Moloney sarcoma and Gross AKR leukemia viruses [9, 11]. Ribavirin also inhibits the development of leukemia in mice induced by Friend, Moloney, Rauscher, and Gross viruses [2, 9, 10, 11]. In the present report the in vitro effects of ribavirin on BLV are examined.

MATERIALS AND METHODS

Cells

Two cell lines were used: BLV-producing fetal lamb kidney (FLK) cells [14] and F-81 cells, a feline sarcoma-positive, leukemia-negative line [13]. Both cell lines were provided by Dr. Janice M. Miller, National Animal Disease Center, Ames, Iowa. The FLK cells were passaged in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml streptomycin, and 100 μg/ml kanamycin, and buffered with 0.19% $NaHCO_3$. These cells are known to have a low level bovine diarrhea virus (BDV) contamination (J.M. Miller, personal communication). The F-81 cells were maintained in the same medium as above with the addition of 0.25% lactalbumin hydrolysate. The same media were used in the antiviral experiments with FBS lowered to 5% in F-81 cell experiments. The FLK cell studies were run in 75 cm^2 tissue culture flasks; studies with the F-81 cells utilized 24-well disposable plastic microplates (Falcon Plastics Division of BioQuest, Oxnard, CA).

Virus

The BLV was produced by the FLK cells and used as supernatant removed from a 36 h monolayer of the cells which had been confluent for 24 h.

Ribavirin

The ribavirin used was obtained from ICN Pharmaceuticals, Inc., Irvine, CA.

Antibody

BLV-positive sheep serum which was negative by complement fixation test for BVD, BVD-positive bovine serum which was negative for BLV, and BLV and BVD-negative sheep serum were obtained from Dr. Janice Miller. The BLV-positive, BVD-negative serum was from a sheep infected experimentally with BLV. More information concerning the sera was reported by Miller and Van der Maaten [7].

Complement fixation test

CF tests for measurements of BLV antigen in tissue culture fluids were run according to the method described by Miller and Van der Maaten [7]. The tissue culture fluids were centrifuged at 66,000 $\times$ *g* for 90 min in a Sorvall rotor, and the pellets resuspended in 0.1% Triton X-100 to concentrate the antigen up to 100-fold.

Indirect immunofluorescence

Syncytia developing in F-81 cells infected with BLV in 24-well microplates were stained by an indirect immunofluorescent technique to confirm that they were induced by BLV. Wells were first stained with BLV-positive or BLV-negative antiserum for 1 h followed by anti-bovine IgG-positive rabbit serum for 1 h. The procedure for handling microplates for immunofluorescence work was described previously [12].

Biochemical cytotoxicity assay

F-81 cells were grown in 24-well disposable microplates, then incubated with ribavirin for 24 h and pulse-labeled 2 h with 2 μCi/ml of the appropriate ^{3}H-labeled precursor. Labeled thymidine, uridine, or leucine were used to quantify DNA, RNA, or protein synthesis, respectively. The TCA-soluble and insoluble fractions were quantified by liquid scintillation. A full description of this method was described previously [12].

RESULTS

In an attempt to eliminate the BLV infection from chronically infected FLK cells, the cells were passaged 8 times (30 days) in the presence of 10, 3.2 or 1 μg/ml of ribavirin. The supernatants from duplicate flasks of treated cells and similarly passaged untreated cells were processed to concentrate the virus, and viral antigen was assayed by CF test. No decrease in titer of viral antigen was seen (Table 1). Cells treated with 3.2 μg/ml ribavirin grew at approximately 60% of the rate of untreated cells. The 10 μg/ml dose level was toxic to the cells when administered to them for this prolonged period.

Stationary monolayers of chronically infected FLK cells were treated for 96 h with varying concentrations of ribavirin and the titers of intra- and extracellular viral antigen subsequently determined. The results, summarized in Table 2, indicate that the amount of detectable antigen was inhibited in direct proportion to the ribavirin concentration, with 4-fold inhibition of extracellular antigen seen at ribavirin levels down to 10 μg/ml; similar reductions of intracellular antigen were seen down to 32 μg/ml.

To determine the effect of ribavirin treatment on newly infected cells, freshly con-

TABLE 1

Effect of long-term[a] treatment with ribavirin on BLV CF antigen production in FLK cells

Ribavirin (μg/ml)	Extracellular CF antigen titer[b]
10	Toxic
3.2	512
1.0	512
0	512

[a] Cells treated for 30 days (8 passages) followed by assay of supernatant fluids for CF antigen.

[b] Titers expressed as reciprocals of highest dilution having positive CF activity.

TABLE 2

Effect of ribavirin on development of BLV CF antigen in FLK cells[a]

Ribavirin concentration (μg/ml)	Visible cytotoxicity[b]	Extracellular CF antigen titer[c]	Intracellular[d] CF antigen titer
1000	++	4	8
320	+	8	16
100	-	32	32
32	-	64	64
10	-	128	128
3.2	-	512	128
1.0	-	512	256
0	-	512	256
		MIC[e]: 10	32

[a] 96 h incubation at 37°C in CO_2 with drug prior to testing; medium changed daily.

[b] ++, Approximately 50% of cells affected, as determined by visual examination and trypan blue dye exclusion; +, ~25% affected cells; -, < 5% discernibly affected cells.

[c] Titers expressed as reciprocals of highest dilutions having positive CF activity. Extracellular antigen obtained by subjecting supernatants to ultracentrifugation at 66,000 × *g* for 90 min, then treating the pellets with 0.1% Triton X-100 in saline.

[d] Intracellular antigen obtained by sonifying washed cells for 1 min, then centrifuging them at 600 × *g* for 5 min and processing the supernatant as described for extracellular antigen.

[e] Minimum inhibitory concentration.

fluent (18 h) monolayers of F-81 cells were exposed to varying concentrations of ribavirin 15 min prior to addition of BLV. After a 96 h incubation, distinct BLV-induced syncytia developed in the cells which were counted in each treatment group. The results of this study (Table 3) indicate that inhibition of syncytia occurred at ribavirin levels as low as 3.2 μg/ml. The syncytia were identified as being BLV-induced by indirect immunofluorescent staining. Visible cytotoxicity, as evidenced by cell abnormalities and trypan blue dye exclusion [6] was seen at 1000 and 320 μg/ml ribavirin levels only, although viable cells were present at these levels in sufficient number for syncytium counts to be made. Using biochemical cytotoxicity parameters, however (Table 4), cytostatic effects evidenced as inhibition of DNA, RNA and protein synthesis occurred at doses down to 1.0 μg/ml.

DISCUSSION

These experiments indicate that the broad-spectrum antiviral, ribavirin, has a moderate but probably non-specific BLV-inhibitory effect in vitro using relatively high dosage levels of the compound applied for short periods of time. No discernible antiviral effect was seen when BLV-infected cells were passaged in the continual presence of low dosage of ribavirin. In the only other published studies on the in vitro effect of ribavirin on onco-

TABLE 3

Effect of ribavirin on development of BLV syncytium formation in F-81 cells[a]

Ribavirin concentration (μg/ml)	Visible cytotoxicity	Average No. of syncytia[b]
1000	++	0
320	+	0
100	-	6
32	-	21
10	-	62
3.2	-	151
1.0	-	198
0	-	197
		MIC: 3.2

[a] 96 h incubation at 37°C in CO_2 with drug prior to assay; medium changed at 48 h.
[b] Per 15 fields in each of 4 microplate cups.

TABLE 4

Effect of ribavirin on uptake of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine in F-81 cells

Ribavirin concentration (μg/ml)	% Drug-free control[a]			
	[^{3}H]thymidine	[^{3}H]uridine	[^{3}H]leucine	
			TCA-insoluble	TCA-soluble
100	58	38	62	64
32	60	26	61	65
10	67	43	75	80
3.2	77	72	76	81
1.0	77	82	86	89

[a] C.p.m., average of 4 samples/dose level.

viruses, Shannon [9], using Gross murine leukemia virus, reported the compound to have a minimum effective dose, defined as that required to produce a 50% inhibition of virus-induced plaques, of 3.2 μg/ml, which was the same minimum effective dosage level determined in the present experiments with BLV. In both studies the ratio of the highest non-cytotoxic concentration of drug (no significant reduction in the host cell multiplication) over the lowest effective drug concentration was considered to be approximately one, a selectivity ratio indicating the antiviral activity to be quite non-selective.

Some uncertainty has existed concerning the association between BLV syncytia formation and BLV replication. By measurement of both syncytium inhibition and reduced development of viral CF antigen, essentially similar results were seen using riba-

virin, suggesting that each parameter used indeed reflects virus quantitation. Further support for this is seen in the fact that other viruses appear similarly inhibited by ribavirin, as determined by other means of measure such as viral cytopathic effects, plaque formation, and intra- and extracellular infectious virus production [11]. Syncytium formation is the only cytopathic change caused by BLV in infected monolayer cell cultures. The CF test used in the studies apparently detects the internal virion polypeptide antigen, although the test may also be demonstrating other BLV antigens as well [3, 16]. Against other RNA viruses, ribavirin's mechanism of antiviral action includes inhibition of viral polypeptide synthesis [11]; assuming the mechanism of action of the compound against RNA tumor viruses is similar, then the use of the CF test for determining antiviral effect would appear to be acceptable. Preliminary studies with the agar gel diffusion test described by Ferrer et al. [4] as a means of detecting viral antigen for our antiviral experiments indicated this test to be markedly less sensitive than either CF test or syncytium count.

The failure of ribavirin, used at low dosages through multiple virus passages, to significantly inhibit BLV could be due either to insufficient levels of the compound being used in the experiment or to the development of a resistant virus. It is probable that the lack of effect is due to inadequate drug rather than resistant virus formation, since previous studies with both an RNA virus (parainfluenza 3) and a DNA virus (herpes virus 1) did not indicate viral resistance to develop readily to ribavirin [11].

Ribavirin has been found to be relatively effective in the treatment of in vivo murine leukemia infections [2, 9, 11], and the possibility of the drug's usefulness for treating infections induced by the bovine virus cannot be excluded. Costs for such experiments may be impractical, however, since large amounts of the compound would be required for treatment of livestock.

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