EFFECT OF RIBAVIRIN ON NORMAL RAT KIDNEY CELLS AND CHICK EMBRYO FIBROBLASTS INFECTED WITH ROUS SARCOMA VIRUS

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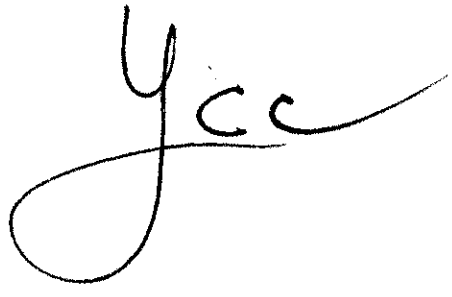
Ribavirin, a synthetic nucleoside, was found to inhibit the replication of Rous sarcoma viruses (RSV) and subsequent cell transformation in chick embryo fibroblasts (CEF). It also blocked the transformation of normal rat kidney (NRK) cells infected with temperature-sensitive mutants of RSV. The action of Ribavirin was found to be reversible as removal of the drug from the NRK cells reversed the effects on cell transformation. Ribavirin appears to have a static effect on cell growth of both NRK and CEF cells. In addition, guanosine, xanthosine and inosine altered the effect of Ribavirin on cell growth.



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CHAPTER I

INTRODUCTION

Ribavirin[1], (1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide) is a synthetic nucleoside that was first synthesized by ICN laboratories in Irvine, California (13). It is reported to have broad spectrum antiviral activity against DNA and RNA viruses, both *in vitro* and *in vivo*. Huffman *et.al*. (8) reported an inhibitory effect by Ribavirin *in vitro* against the following viruses: adenotype 3, herpes types 1 and 2, myxoma, cytomegalo, vaccinia, parainfluenza types 1 and 3, subacute sclerosing panencephalitis, Newcastle disease and measles. Inhibition *in vivo* is reported by Sidwell (12) against Friend leukemia virus in mice, and by Potter (15) against influenza virus in ferrets.

A search of the literature reveals no reports on the effect of Ribavirin against cell transformation. In this study, the effect of Ribavirin on cellular transformation of rat kidney cells (NRK) and chick embryo fibroblasts (CEF) infected with different wild type or temperature-sensitive (ts) mutants of the Rous sarcoma virus was

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[1]Also called Virazole.

investigated.

The Rous sarcoma virus (RSV) is an RNA tumor virus belonging to the Retrovirus family. It is an enveloped type C oncovirus with a single-stranded RNA genome. The RNA tumor viruses replicate by way of a DNA intermediate which uses a virion RNA dependent DNA polymerase (17).

The genome of the RNA tumor virus contains four genes. The first gene is the "gag" gene, which stands for group specific antigens. This gene codes for the major internal structural proteins. The second gene is the "pol" gene or polymerase gene. It codes for the RNA dependent DNA polymerase which is more commonly referred to as the reverse transcriptase (4). Next comes the "env" or envelope gene. This gene is responsible for coding the envelope glycoproteins of the tumor viruses. The last gene is the "src" or sarcoma gene. This gene is responsible for the oncogenic transformation induced by the RNA sarcoma viruses (17).

Host cell transformation is manifested by several parameters. The major and most common parameter is a rounding of cellular morphology. The typical transformed cell has a round, grape-like appearance when compared to the long slender morphology of normal cells. The transformed cell shows an increased growth rate with a loss of contact inhibition of movement resulting in a high saturation density (19). Other parameters of transformed cells

include an increase in sugar transport (9), colony formation in an agar suspension (3) and low levels of cyclic AMP (1).

The different parameters of transformation are believed to be caused by the presence of a transforming protein which is coded for by the "src" gene (9). The theory of the transforming protein is supported by the observation that protein synthesis inhibitors, such as cyclohexamide or puromycin, can cause transformed cells to revert to normal phenotype (2). Isolation and purification of this protein has yet to be performed. Evidence of the transforming protein is further supported by temperature-sensitive RSV mutants which have a defect in the "src" gene. These mutants, called class T-1 mutants, lose their transformed phenotype when shifted to the nonpermissive temperature and regain it on shifting back to the permissive temperature. The specific permissive and nonpermissive temperatures vary according to the mutants and cell lines used. Although class T-1 mutants fail to maintain their transformed phenotype at the nonpermissive temperature, they still produce viral progeny in the permissive host cells (20).

The natural host of the Rous sarcoma virus is the chick embryo fibroblast. Transformation studies performed on the chick cell are complicated by the production of

viral progeny. The release of the progeny from the chick cell results in plasma membrane loss as the envelope of the virus is derived from the plasma membrane. Prolonged viral production therefore results eventually in cell lysis.

The use of a heterologous host instead of the homologous host (i.e. chick embryo fibroblast) eliminates the interference produced by viral progeny production. Mammalian cells infected with RNA tumor viruses exhibit only the cellular transormation and do not produce viral progeny (5). Therefore cell lines can be developed as excellent model systems of cellular transformation by RNA tumor viruses. In this study, normal rat kidney (NRK) cells infected with either a temperature-sensitive mutant or wild type RSV were used. At the permissive temperature (33°C), the ts-mutant infected NRK cells exhibit the morphological growth characteristics of the transformed state, while at the nonpermissive temperature (39°C), they exhibit the normal characteristics. NRK cells infected with the wild type RSV exhibit the transformed phenotype at either temperature (5).

Since there have been no published reports on the effect of Ribavirin on cellular transformation, we have investigated its effects on the transformation of chick embryo fibroblasts and normal rat kidney cells infected with either temperature-sensitive mutants or wild type

Rous sarcoma viruses. We have looked at the effects of Ribavirin on cell growth of NRK and CEF cells, in order to determine if there is any cytotoxicity towards the cells. Also, additional experiments were performed in order to determine any correlation between Ribavirin's site of action in cellular transformation as compared to results obtained with viral replication (10,14).

CHAPTER II

METHODS AND MATERIALS

Cell Lines

The cell lines used were primary chick embryo fibroblasts (CEF), normal rat kidney (NRK) cells, and NRK cells infected with either a temperature-sensitive mutant LA31, or B77 a wild type RSV. These cell lines, maintained in this laboratory, were originally obtained from Peter Vogt's laboratory in California.

Primary chick embryo fibroblasts of c/o phenotype were prepared directly from fertile eggs (SPAFAS, Norwich, Conn.) according to published techniques (16). All cells were grown in F-10 (Ham's) media with Hank's salts (see Appendix for composition), supplemented with 5 per cent cadet calf serum, 10 per cent tryptose phosphate broth, penicillin (100 units/ml) and streptomycin (100 ug/ml). The cells were grown in humidified 5 per cent CO_2 incubators. The F-10 media was obtained from GIBCO in Berkeley Calif.

Viruses

The following strains of wild type Rous sarcoma viruses were used: Prague-A (PR-A), Schmidt-Ruppin-D (SR-D) and Bratislava-77 (B-77). A mutant of the PR-A

strain was also used, LA-23. These viruses were obtained from Peter Vogt's laboratory in California (7).

Ribavirin

Ribavirin, (1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide) was obtained from ICN laboratories, Irvine Calif., with the help of Dr. Lois B. Allen. Dilutions of the drug were made with the culture medium described above.

Experimental

Chick Embryo Fibroblast Transformation

In each case 1.2×10^6 CEF cells were seeded into 60mm tissue culture plates (Corning, Corning, New York) containing culture media and varying concentrations of Ribavirin. Approximately 1.2×10^5 FFU of the different RSV viruses (multiplicity of infection-0.1) were also seeded onto the cells. Two plates per concentration per virus were prepared. The concentrations of Ribavirin used were 10, 75, 100 and 500 ug/ml. A set of double controls was used. One set contained CEF cells and virus without Ribavirin. The second set contained CEF cells without virus or Ribavirin. The cells infected with wild type RSV were incubated at 39°C. The cells infected with the LA23 mutant were incubated at the permissive temperature (33°C). They were grown for six days or until transformation was complete in the

control cells. The degree of transformation in each plate was then noted and the medium containing the viral progeny removed. Transformation was graded according to the relative percent of transformed cells present. Grading was on a scale of 0-10, with 0 being no transformed cells present and 10 being all cells transformed. The virus harvests were then used in the focus assay experiments.

RSV Focus Assay

Dilutions of each virus harvest were prepared and sonicated to destroy the host cells. Duplicates of each harvest were also prepared. In each case $1x10^6$ CEF cells were seeded into 60mm tissue culture plates containing culture media plus 0.5 per cent dimethylsulfoxide (DMSO). Next 0.1 ml of each dilution was inoculated into the corresponding plate. The cells were then incubated overnight at the same temperatures as before. The following morning, the medium from each plate was removed and replaced with 5.0 ml of overlay medium. The composition of the overlay medium is shown in the Appendix. Three days later, a second overlay was performed, followed by a third overlay two days after the second overlay. Following the third overlay, the foci from each plate were counted.

Effect on Transformation at Permissive (33°C) Temperature

Using the LA31-infected NRK cells, 1.2×10^6 cells were seeded into 60 mm plates and allowed to grow at 33°C until confluency and transformation were uniform throughout the plates. The medium was then changed for medium containing Ribavirin at concentrations of 10, 50, 75, 100, 500 and 1,000 ug/ml. A set of control plates with no Ribavirin present was also prepared. The cells were examined every day for six consecutive days, observing the degree of transformation and saturation density. During the six day observation period, the medium was changed once for fresh medium containing the drug.

Effect on Transformation After Drug Removal

At the completion of the six day interval, the cells were washed with regular medium to remove the drug and then placed in fresh medium without the drug. The cells were again observed once a day for reversion from fibroblast to the transformed state. The cells were observed for one week with one medium change.

Effect on Transformation From Nonpermissive (39°C) to Permissive (33°C) Temperature

Cells were grown as before in the permissive temperature experiment, except for the incubation temperatures. After seeding the plates, the cells were grown at 33°C overnight and then shifted to 39°C. Once the cells had

reached confluency and reverted to normal phenotype, the medium was changed for media containing Ribavirin at concentrations of 10, 75, 100 and 500 ug/ml. The cells were then shifted to the permissive temperature (33°C) and observed once a day for one week and the degree of transformation noted.

Effect of Ribavirin on Cell Growth of Control CEF, NRK and B77-NRK Cell Lines

In each case 35 mm tissue culture plates were seeded with $2x10^5$ cells per plate. The concentrations of Ribavirin used were 10, 50, 75 and 100 ug/ml. Two plates for each concentration were used for each day. The cells were counted for seven consecutive days. Ribavirin was added at the same time that the cells were seeded. Each day the two plates for each concentration were trypsinized with 1.0 ml of 0.4 per cent trypsin and the cells gently scraped off with a rubber policeman. All cell counts were performed using an automated Hycell cell counter.

Effect of Ribavirin on Cell Growth of B77-NRK Cells in the Presence of Guanosine, Xanthosine or Inosine

In each case $2x10^5$ cells were seeded into 35 mm plates containing varying concentrations of Ribavirin and 200 ug/ml of either guanosine, xanthosine or inosine. The concentrations of Ribavirin used were 10, 75, 100 and 500 ug/ml. A set of double controls was used. One set contained the

cells and Ribavirin without nucleoside while the other set contained only the cells without Ribavirin or nucleoside. The cells were counted as in the previous experiment.

CHAPTER III

RESULTS

The effect of Ribavirin on CEF transformation and subsequent RSV replication are shown in Table I.

TABLE I

EFFECT OF RIBAVIRIN ON TRANSFORMATION AND REPLICATION IN CEF C/O CELLS

Ribavirin Conc.	LA-23		PR-A		B-77		SR-D		Control	
	TFO*	FFU**	TFO	FFU	TFO	FFU	TFO	FFU	TFO	FFU
Control	4	660	9	3.9×10^4	8	1.3×10^4	9	8.1×10^3	0	0
10 ug/ml	0	40	0	50	1	480	0	0	0	0
50 ug/ml	0	0	0	0	0	60	0	0	0	0
75 ug/ml	0	0	0	0	0	0	0	0	0	0
100 ug/ml	0	0	0	0	0	0	0	0	0	0

*TFO-degree of transformation at time of virus harvest

**FFU-focus forming units reported as FFU/ml

In comparing the control cultures to the experimental, it is evident that Ribavirin at concentrations of 10, 50, 75 and 100 ug/ml completely blocked cellular transformation. The only exception noted was the B77 strain at a concentration of 10 ug/ml of Ribavirin. These plates exhibited less than 10 per cent total transformation. A focus assay

performed on the virus harvests showed a 90 per cent or greater inhibition of viral production when compared to the control cells. Again, the B77 strain showed greater resistance to Ribavirin, having 60 FFU/ml with 50 ug/ml of Ribavirin, and 480 FFU/ml with 10 ug/ml of Ribavirin.

The results of experiments on Ribavirin's effect on cellular transformation of LA31- and B77-infected NRK cells are shown in Tables II, III and IV. Table II shows the effects of Ribavirin on LA31-infected NRK cells that were grown at the nonpermissive temperature (39°C), introduced to Ribavirin, and then shifted to the permissive temperature (33°C).

TABLE II

EFFECT OF RIBAVIRIN ON TRANSFORMATION OF LA31-INFECTED NRK CELLS GROWN AT 39°C AND SHIFTED TO 33°C

Ribavirin Conc.	Degree of Transformation Time (Hours) 0	64	87	112	138	162
Control	2	2	5	7	8	9
10 ug/ml	2	2	2	2	2	2
75 ug/ml	2	1	2	1	1	1
100 ug/ml	2	1	2	1	1	1
500 ug/ml	2	1	2	1	1	1

The retransformation from normal phenotype to transformed phenotype was completely blocked with every concentration of Ribavirin used. The LA31-infected NRK cells were then grown at the permissive temperature (33°C) so that the transformed phenotype was exhibited. The results of the addition of Ribavirin to these cells are shown in Table III.

TABLE III

EFFECT OF RIBAVIRIN ON TRANSFORMATION OF LA31-INFECTED NRK CELLS GROWN AT 33°C

	Degree of Transformation						
	Time (Hours)						
Ribavirin Conc.	0	24	50	68	100	119	140
Control	10	10	10	10	10	10	10
10 ug/ml	10	10	9	9	9	8	7
50 ug/ml	7	7	6	5	5	5	5
75 ug/ml	7	7	6	5	4	4	4
100 ug/ml	10	8	5	4	3	3	3
500 ug/ml	10	8	6	4	4	3	3
1000 ug/ml	10	8	5	4	4	3	3

The 100 ug/ml concentration proved to be the most effective in causing the cells to revert from the transformed phenotype to the normal phenotype at the permissive temperature. There was a 70 per cent reduction from the transformed to

the normal phenotype. Those concentrations greater than 100 ug/ml exhibited an increased cytotoxicity and loss in cell sheath confluency. The 100 ug/ml concentration did not show cytotoxicity while causing the morphological reversion. Concentrations of 75 ug/ml and less did not exhibit as great an effect on cell transformation. In addition, when Ribavirin was removed from the cells and replaced with regular culture medium, the cells which had reverted to the normal phenotype, changed back to the transformed phenotype within one week. The effects of Ribavirin on B77-infected NRK cells are shown in Table IV.

TABLE IV

EFFECT OF RIBAVIRIN ON TRANSFORMATION OF B77-INFECTED NRK CELLS GROWN AT 33°C

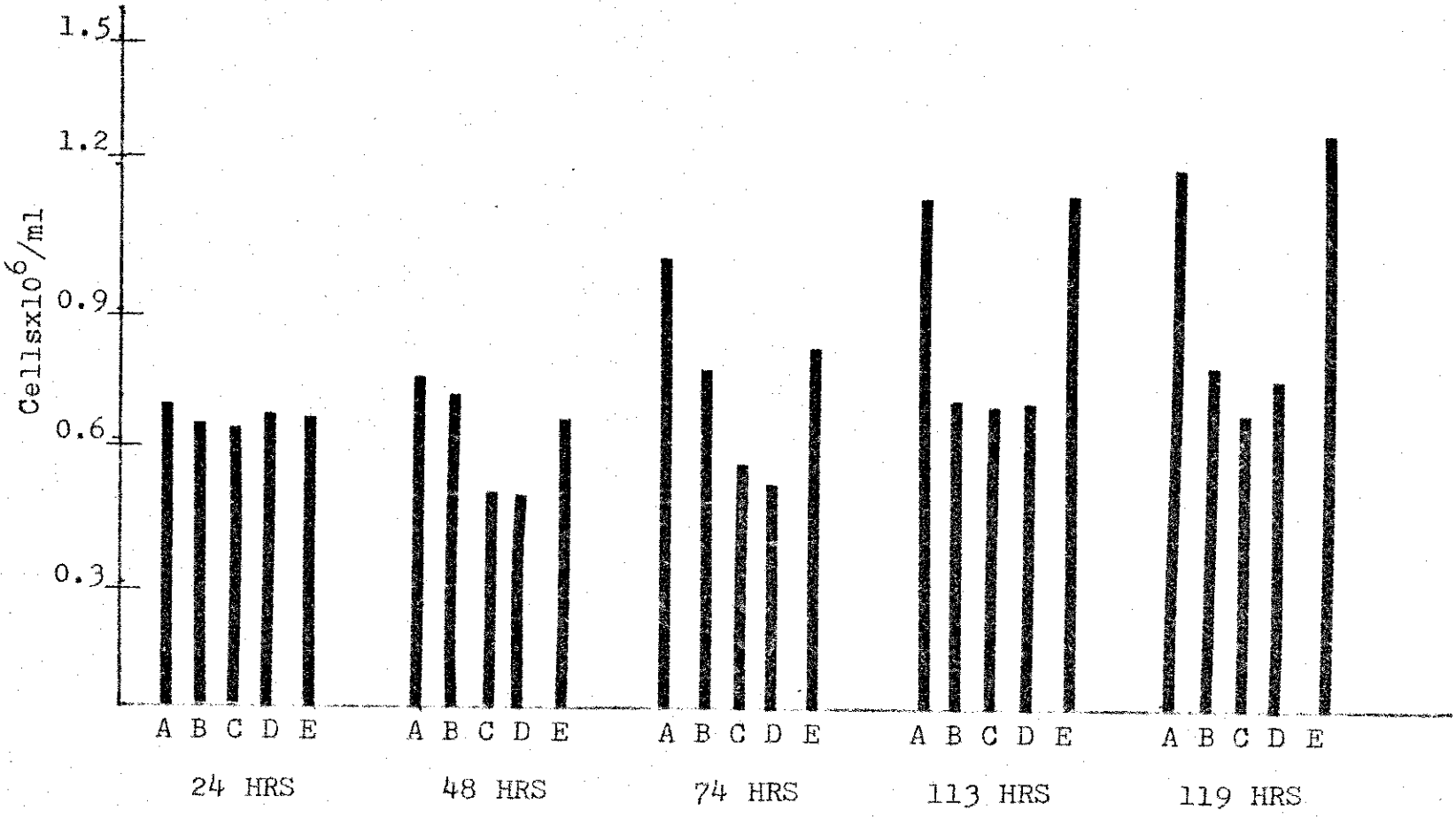
	Degree of Transformation						
	Time (Hours)						
Ribavirin Conc.	0	24	50	68	100	119	140
Control	10	10	10	10	10	10	10
10 ug/ml	10	10	10	10	10	9	9
50 ug/ml	8	8	8	8	8	8	8
75 ug/ml	7	8	7	7	8	8	8
100 ug/ml	10	10	10	9	9	9	9
500 ug/ml	10	9	10	9	9	9	8
1000 ug/ml	10	10	10	9	9	9	9

B77 is a wild type virus, so that the infected NRK cells always exhibit the transformed phenotype. They do not show temperature dependence. As Table IV shows, Ribavirin had no noticeable effect on cellular transformation. At the 100 ug/ml concentration, there was only a 10 per cent reduction compared to 70 per cent with the LA31-infected NRK cells.

Ribavirin's effect on cell growth was studied with three cell lines: CEF, NRK and B77-infected NRK. Figures 1,2 and 3 show the results of these experiments. At a concentration of 10 ug/ml, there was a very slight difference in cell growth compared to the control cells in all three cell lines. All the other concentrations however, appeared to have an inhibitory effect on cell growth. That is to say, the cell numbers remained fairly constant throughout the experiment. Concentrations greater than 100 ug/ml showed a cytopathic effect and an accumulation of cellular debris.

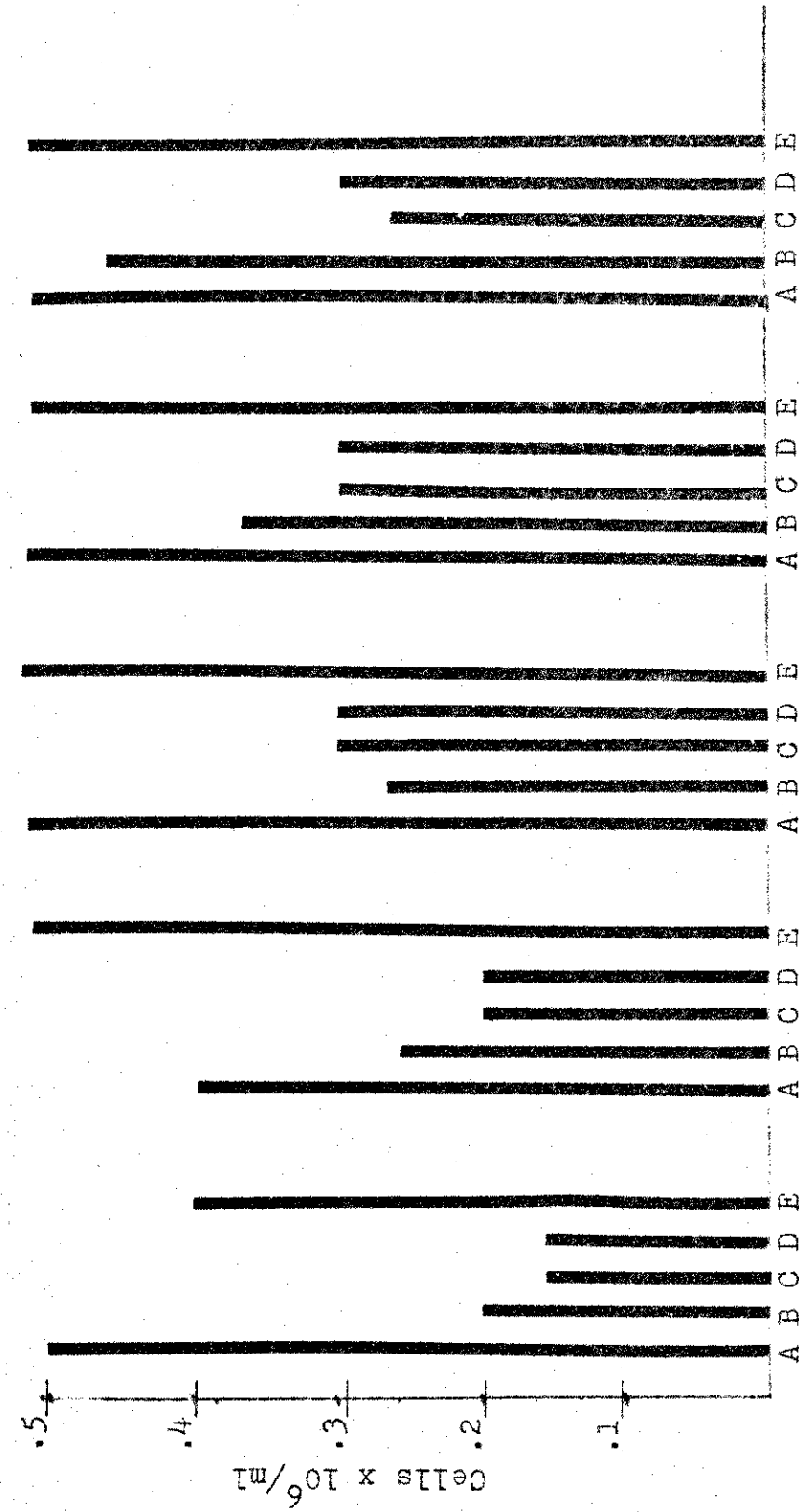
The effects of the addition of guanosine, xanthosine or inosine on cell growth of B77-infected NRK cells treated with Ribavirin was also studied. These results are shown in Figures 4, 5 and 6. The addition of 200 ug/ml of guanosine greatly inhibited the effect of Ribavirin on cell growth. Xanthosine showed a marked increase in inhibition of the effect of Ribavirin, but not as great as guanosine.

The addition of inosine also altered the effect of Ribavirin, however it too was not as great as the effects seen with guanosine. None of the nucleosides completely inhibited the action of Ribavirin.



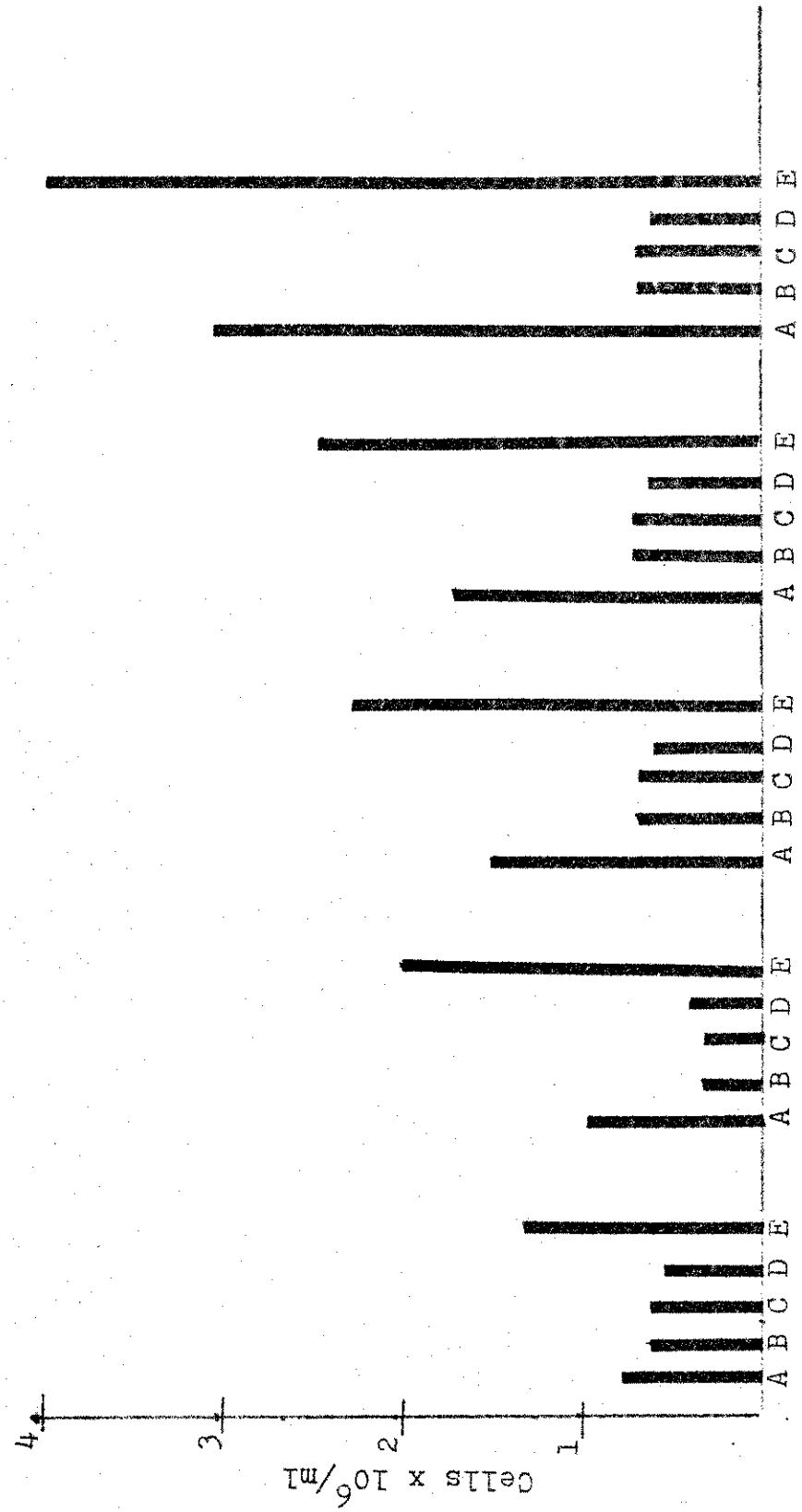
A=10 ug/ml B=50 ug/ml C=75 ug/ml D=100 ug/ml E=Control

Fig. 1--The effect of Ribavirin on CEF cell growth



A=10 ug/ml B=50 ug/ml C=75 ug/ml D=100 ug/ml E=Control

Fig. 2--The effect of Ribavirin on NRK cell growth



A=10 ug/ml B=75 ug/ml C=100 ug/ml D=500 ug/ml E=Control

Fig. 3--The effect of Ribavirin on B77-infected NRK cell growth

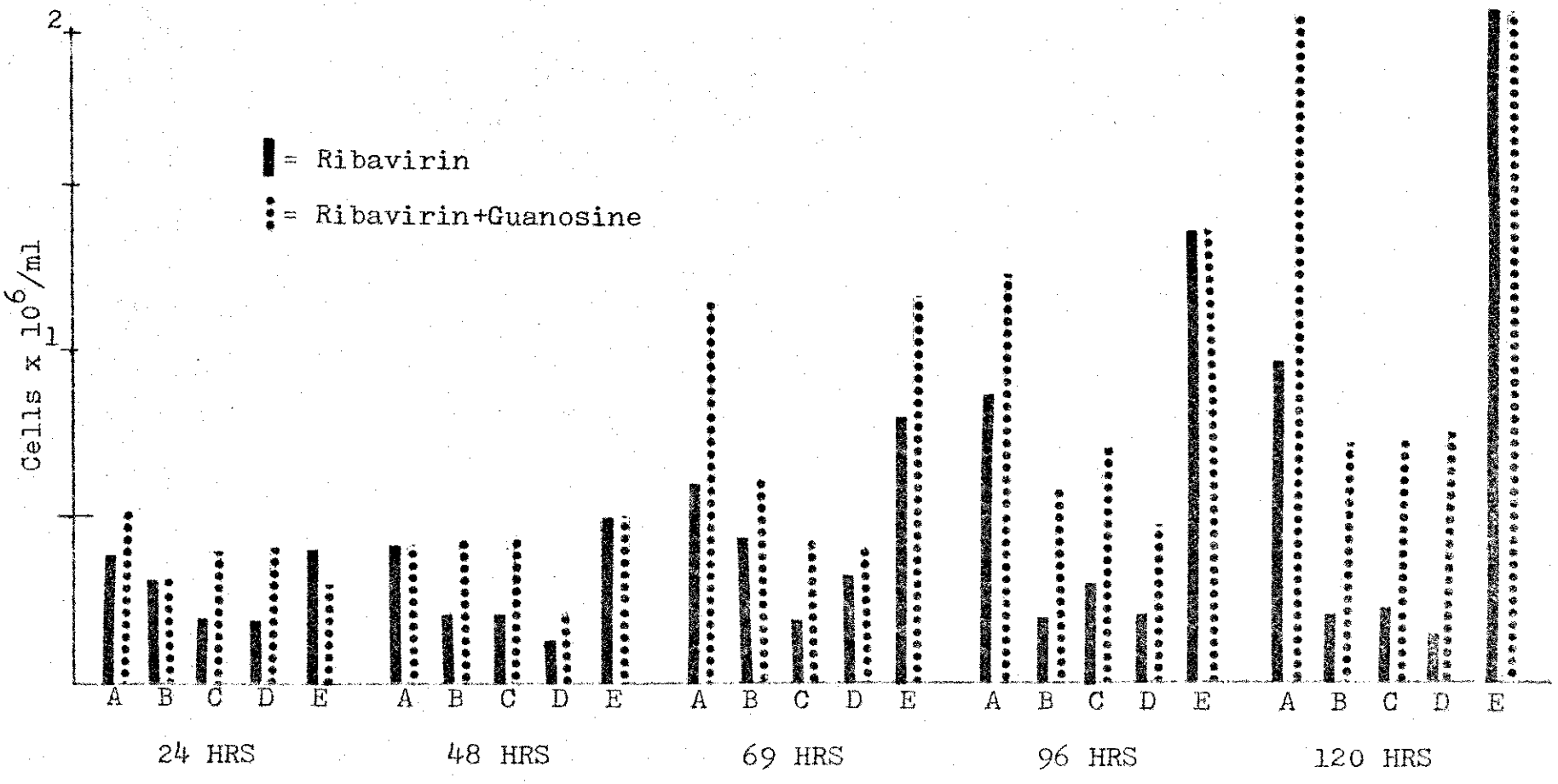
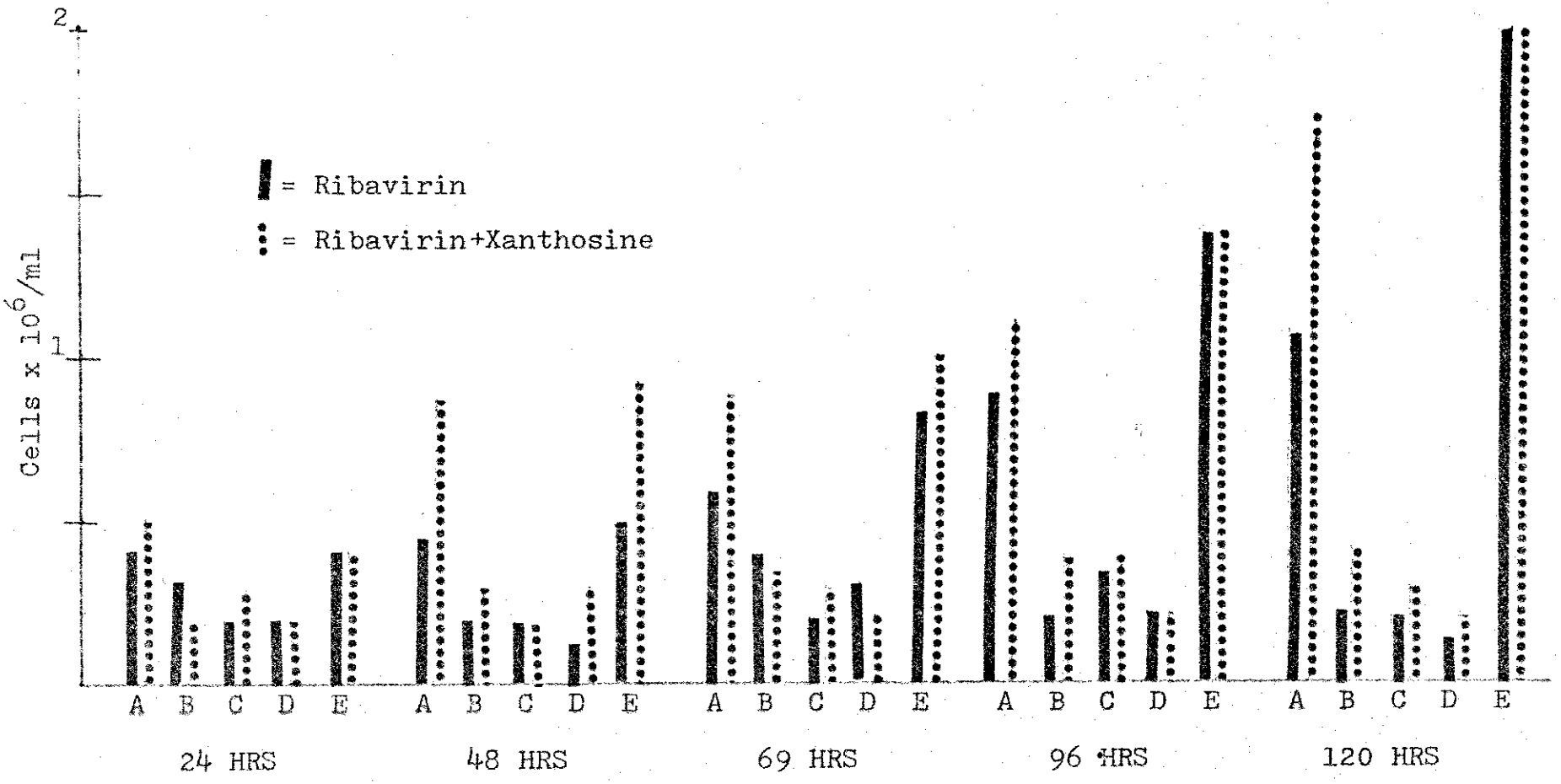
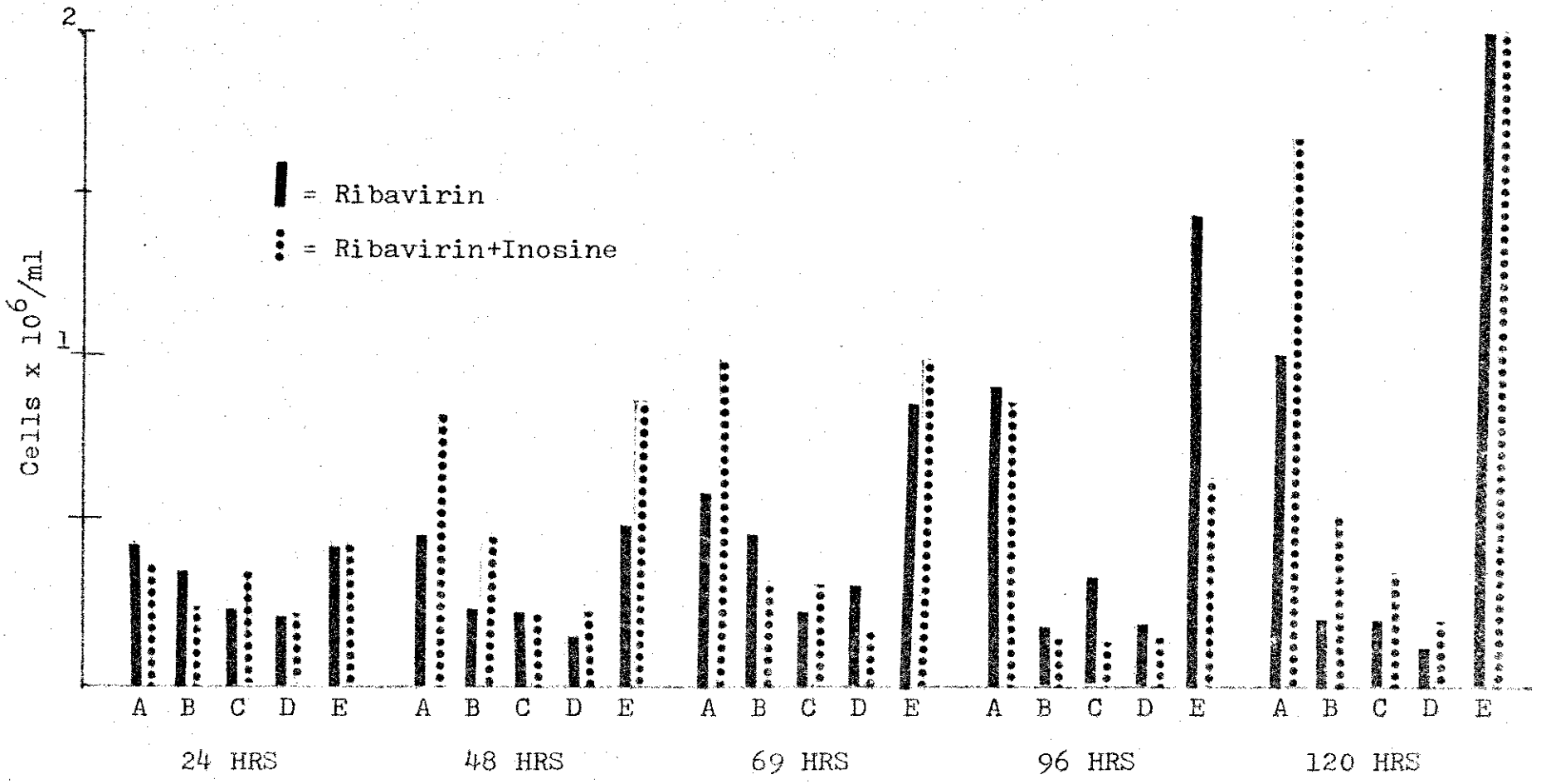


Fig. 4--A comparision on the effect of Ribavirin and Ribavirin + Guanosine on B77-infected NRK cell growth



A=10 ug/ml B=75 ug/ml C=100 ug/ml D=500 ug/ml E=Control

Fig. 5--A comparision on the effect of Ribavirin and Ribavirin + Xanthosine on B77-infected NRK cell growth



A=10 ug/ml B=75 ug/ml C=100 ug/ml D=500 ug/ml E=Control

Fig. 6--A comparision on the effect of Ribavirin and Ribavirin + Inosine on B77-infected NRK cell growth

CHAPTER IV

DISCUSSION

Ribavirin clearly inhibits the replication of Rous sarcoma viruses in the chick cell. It also has a definite effect on cellular transformation of both chick embryo fibroblasts (CEF) and normal rat kidney cells (NRK). The studies on transformation of NRK cells showed a resistance of the B77 wild type infected NRK cells to the action of Ribavirin. The B77 strain was also more resistant to Ribavirin in the chick cell system. There are two possible explanations for this resistance. It is possible that in the NRK system, the wild type genome is much more stable and therefore resistant to Ribavirin. But since B77 was also more resistant in the CEF system than the other wild type RSV, it is possible that B77 is just a much more resistant strain. The PR-A strain was not resistant in the CEF system and LA31 is a mutant of the PR-A strain. Unfortunately, we do not have a wild type PR-A infected NRK cell line to study.

Kawai (9) and Ash (2) have shown that protein synthesis inhibitors such as cyclohexamide and puromycin, caused a reversion from the transformed phenotype to normal phenotype in cells infected with a temperature-

sensitive RSV. In addition, actinomycin-D, a RNA synthesis inhibitor, has also been found to cause this morphological reversion (Y.C. Chen, 1979, unpublished data). These results would indicate that viral directed transcription and translation are necessary for the maintenance of the transformed state. They also support the hypothesis that the "src" gene codes for a transforming protein which, in class T-1 mutants, is irreversibly inactivated at the nonpermissive temperature and needs to be resynthesized upon the shift to the permissive temperature in order to produce retransformation (18). Any interruption of viral directed transcription or translation would directly affect the production of the transforming protein. Interruption of this protein would then cause the reversion of cell morphology and other transformation parameters. The pronounced reversion or inhibition of cell transformation by Ribavirin in cells infected with temperature-sensitive RSV mutants, would indicate that Ribavirin interferes with viral directed transcription, thereby disrupting the production of the transforming protein.

Previous papers by Streeter et.al. (14) and Lowe et.al. (10) indicated that the mechanism of action of Ribavirin is in the guanine synthesis pathway. It appears to be a competitive inhibitor of inosine monophosphate (IMP) dehydrogenase (10). Once Ribavirin has entered the

cell, it becomes phosphorylated. The monophosphate is the inhibitor of the cellular IMP dehydrogenase. In addition, the monophosphate can be phosphorylated further to the triphosphate. The triphosphate form has been shown to be a competitive inhibitor to the purines ATP and GTP. It is a specific selective inhibitor of a viral polymerase (11). Therefore Ribavirin can be looked upon as having two major sites of action. One is viral specific while the other is not.

It would seem reasonable then to theorize how Ribavirin causes the reversion of cell transformation. By interrupting guanine synthesis, the transcription of viral RNA would be interrupted. Also, if Ribavirin were phosphorylated to the triphosphate form, the viral polymerase associated with transcription would be blocked. Both mechanisms would result indirectly in affecting the production of the transforming protein, thereby causing the cell to lose its transformed state.

Since the inhibition of IMP dehydrogenase would affect both viral and cellular mechanisms, Ribavirin should have some effect on host cell growth. This theory corresponds to our findings with cell growth experiments. Ribavirin had a definite static effect on cell growth, indicating a possible shutdown at some site of host cell metabolism. The theory of the site of action being at IMP dehydrogenase was further supported by our studies with the nucleosides

guanosine, xanthosine and inosine. In the guanine synthesis pathway, inosine is converted to xanthosine by IMP dehydrogenase. Xanthosine is then eventually converted to guanosine. So the addition of either guanosine or xanthosine to culture media should help to bypass the action of Ribavirin. The addition of inosine would cause the competitive inhibition by Ribavirin to be decreased as a higher concentration of inosine would be present. Our results support these theories. The addition of guanosine, xanthosine or inosine altered the effect of Ribavirin on cell growth.

Ribavirin is currently being used for human treatment in both Europe and Mexico (L.B. Allen, 1979, personal communication). Douglas et.al. (6) has shown that treatment with 1,000 mg of Ribavirin orally per day, reduces both symptoms and viral numbers in influenza A infection in human volunteers. Ribavirin is currently under study by the Federal Drug Administration for possible licensing in the U.S. This is being delayed however, by the fact that Ribavirin has been found to be terratogenic in rodents (L.B. Allen, 1979, personal communication). Ribavirin has potential as an antiviral agent. In addition to its antiviral effect, further research should also investigate a possible role of Ribavirin in the treatment of virus related tumors.

APPENDIX

COMPOSITION OF F-10 MEDIA WITH HANK'S SALTS

Inorganic Salts	mg/l
$CaCl_2$ (anhyd)	33.29
$CuSO_4$ $5H_2O$	0.0025
$FeSO_4$ $7H_2O$	0.834
KCl	285.0
KH_2PO_4	83.0
$MgSO_4$ (anhyd)	74.64
NaCl	7400.0
Na_2HPO_4 (anhyd)	153.7
$ZnSO_4$ $7H_2O$	0.0288
Other Components	
Glucose	1100.0
Hypoxanthine (Na salt)	4.68
Lipoic Acid	0.2
Phenol Red	1.2
Sodium Pyruvate	110.0
Thymidine	0.7
Amino Acids	
L-Alanine	9.0
L-Arginine HCl	211.0
L-Asparagine H_2O	15.01
L-Aspartate	13.0
L-Cystine	25.0
L-Glutamate	14.7
L-Glutamine	146.0
Glycine	7.51
L-Histidine HCl H_2O	23.0

Amino Acids Con't.	mg/l
L-Isoleucine	2.6
L-Leucine	13.0
L-Lysine HCl	29.0
L-Methionine	4.48
L-Phenylalanine	5.0
L-Proline	11.5
L-Serine	10.5
L-Threonine	3.57
L-Tryptophan	0.6
L-Tyrosine (disodium salt)	2.62
L-Valine	3.5
Vitamins	
Biotin	0.024
D-Ca Pantothenate	0.715
Choline Cl	0.698
Folic Acid	1.320
I-Inositol	0.541
Niacinamide	0.615
Pyridoxine HCl	0.206
Riboflavin	0.376
Thiamine HCl	1.0
B_{12}	1.360

COMPOSITION OF OVERLAY MEDIUM

Double Strength F-10	100.0 ml
Tryptose Phosphate Broth	25.0 ml
Cadet Calf Serum	12.5 ml
Chick Serum	1.0 ml
DMSO	0.5 ml
0.7% Agar	100.0 ml
Penicillin	100 units
Streptomycin	100 ug

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