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EFFECT OF RIBAVIRIN ON ACUTE AND PERSISTENT HUMAN CORONAVIRUS INFECTION, IN VITRO

by

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for
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ABSTRACT

The antiviral effect of ribavirin was tested on human coronavirus persistently infected cells (HV) and on L132 cells acutely infected with HCV 229E and VH virus. Visible cytotoxicity was minimal in all cell systems even at 500 ug/mL at 72 h post-treatment. However, there was pronounced cytostatic effect with the 50% inhibitory concentration in all cases being greater than 100 ug/mL ribavirin.

Ribavirin markedly reduced the yields of virus produced during both the 229E and VH acute infections as well as in the HV persistent infection. There was a direct dose-response relationship between the concentrations of ribavirin and the yield of virus and near maximum reduction in the total PFU/cell ratios was achieved with 50 ug/mL.

The relationship between input virus and antiviral activity in an acute infection was studied using the plaque reduction assay. Ribavirin inhibited plaque formation by both 229E and VH viruses with a maximum effect occurring at 50 ug/mL. The size of plaques was also much smaller when higher concentrations of ribavirin and higher input virus was involved.

Guanosine (200 ug/mL) was effective in counteracting the antiviral activity of ribavirin only in the 229E and VH acute infection even though guanosine alone could inhibit normal cell replication. In the plaque reduction assay of both viruses, guanosine alone inhibited plaque formation and in tests with ribavirin any counteraction was poorly defined. In HV cells, guanosine inhibited both cell replication and virus production to the same extent as ribavirin (50 ug/mL).

Attempts to cure the HV cells of their persistent infection by continuous passage of the cells in the presence of ribavirin was

initially encouraging. By the third cell passage with 50 ug/mL ribavirin, virus production seemed to be eliminated. But low levels of virus appeared while still in the presence of 50 ug/mL ribavirin at cell passages 5-7. When the drug was removed, virus production resumed normal levels within 24 h. The emergence of possible ribavirin resistant strains was of great interest and requires further study.

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I. LITERATURE REVIEW

Currently there are two main areas of research into the control of viral diseases. The first is aimed at protecting the host by manipulating the immune system to develop an immunity specific for the disease in question. Although such an approach has had some spectacular successes, many viral infections cannot be controlled in this way. The second area of viral disease control has been the development of chemicals capable of acting to interfere with and/or prevent infection by viruses, thus limiting the spread of the infectious agents. Several antiviral agents are presently available, however, their effect on diseases caused by coronaviruses has not been well documented either in vitro or in vivo.

Our interest lay in the use of one of these agents - ribavirin - as a possible means of curing a cell line persistently infected with a human coronavirus, strain 229E (HCV/229E). In order to evaluate the potential of ribavirin for this purpose, however, it was first necessary to determine the effect of the drug on the virus. This study was designed to provide the necessary information.

1. CORONAVIRUS

The coronaviruses were first recognized and morphologically defined as a separate group of viruses by Tyrrell and co-workers(1968). Since then, it has been well recognized that coronaviruses are responsible for a number of diseases of clinical and economic importance, in particular respiratory and gastrointestinal disorders. Table 1 lists the coronaviruses described to date, their natural hosts, and their associated diseases.

Table 1. Currently recognized Coronaviruses, their natural hosts, associated diseases and antigenic groups.

Antigenic group	Virus	Host	Disease
I	HCV-229E	Human	Respiratory disease
	TGEV	Pig	Enteritis
	CCV	Dog	Enteritis
	FECV	Cat	Enteritis
	FIPV	Cat	Respiratory disease, enteritis, hepatitis, neurologic disease, and others.
II	HCV-OC43	Human	Respiratory disease
	MHV	Mouse	Hepatitis, encephalomyelitis, enteritis, vasculitis.
	HEV	Pig	Vomiting and wasting disease, encephalomyelitis.
	BCV	Cattle	Enteritis
	RbCV	Rabbit	Enteritis
III	IBV	Chicken	Respiratory disease
IV	TCV	Turkey	Enteritis
Unclassified	HECV	Human	Enteritis?

Abbreviations:

BCV.....Bovine coronavirus
CCV.....Canine coronavirus
FECV.....Feline enteric coronavirus
FIPV.....Feline infectious peritonitis virus
HCV.....Human coronavirus
HECV.....Human enteric coronavirus
HEV.....Haemagglutinating encephalomyelitis virus
IBV.....Infectious bronchitis virus
MHV.....Murine hepatitis virus
RbCV.....Rabbit coronavirus
TCV.....Turkey coronavirus
TGEV.....Transmissible gastroenteritis virus

Adapted from Holmes (1985).

Coronaviruses are of interest because of their narrow host range and the documented capacity of several to produce persistent infections in vivo (MHV in mice) and in vitro (MHV in murine cell cultures, and HCV 229E in human cell cultures). Because there is no available immunological method to control coronavirus infections at this time, the development of more potent and more selective antiviral agents for clinical use has become a neccesity.

i. Properties of coronaviruses.

Coronaviruses are a group of pleomorphic enveloped RNA-containing, ether-labile viruses. They have a diameter ranging from 60 to 220 nm with characteristic large, sometimes widely spaced, 12 to 24 nm long spikes or peplomers that form a corona around the particle and provide the name for this virus family.

The genomic RNA is an infectious single-stranded molecule which is capped and polyadenylated. The molecular weight is $5\text{-}7\times10^{6}$ which corresponds to about 15,000-20,000 nucleotides. There is no extensive sequence reiteration in the coronavirus genome. Coronavirions characteristically have three types of protein; a phosphorylated nucleocapsid protein (MW. $50\text{-}60\times 10^{3}$), which is complexed with the genome as a helical ribonucleoprotein (RNP); an N-glycosylated surface peplomer protein (E2), associated with glycopolypeptides of $90\text{-}180\times10^{3}$ molecular weight, which is acylated and is responsible for virus attachment and cell-to-cell fusion (this protein may be removed by protease treatment); and a transmembrane matrix protein (E1), associated with polypeptides of molecular weight $20\text{-}35\times10^{3}$ which have variable degrees of glycosylation, either O- or N-linked (MHV; IBV) (reviewed by Siddell et al., 1983). Figure 1 shows a schematic model of the

FIGURE 1

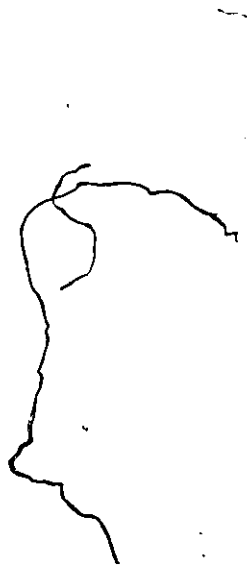
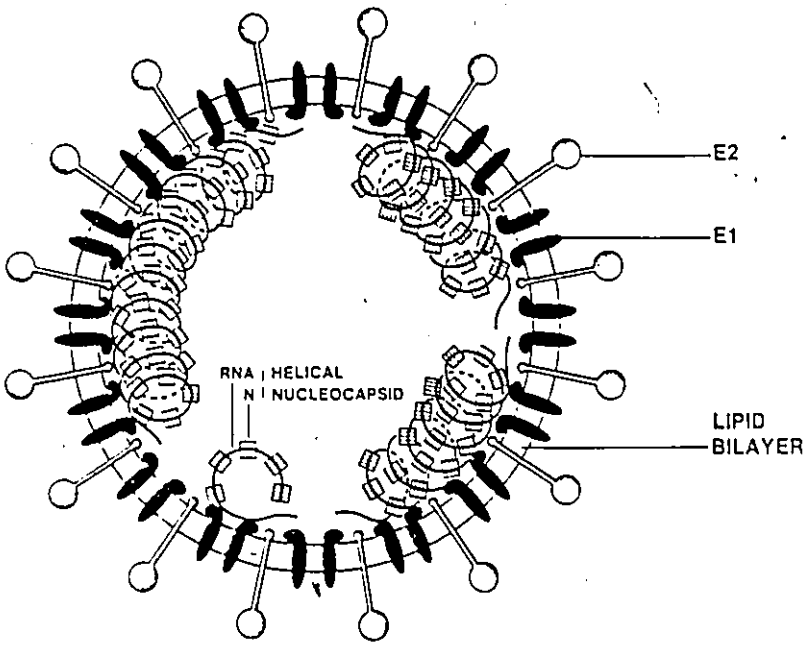


Figure 1. Structural model of a coronavirion.

The viral nucleocapsid is a long, flexible helix composed of the plus-strand genomic RNA and many molecules of the phosphorylated nucleocapsid protein, N. The viral envelope includes a lipid bilayer derived from intracellular membranes of the host cell and two viral glycoproteins, E1 and E2. The peplomers are composed of E2. The matrix glycoprotein E1 penetrates through the lipid bilayer and interacts with the nucleocapsid within the virion.

Taken from Holmes (1985).


E2
E1
RNA | HELICAL
N | NUCLEOCAPSID
LIPID
BILAYER

coronavirion and the relationship of its RNA and protein components (Holmes, 1985).

Coronaviruses can cause either severe or moderate infections of cells, depending on the virus strain and the host-cell type (Holmes, 1985; Sturman and Holmes, 1983; Wege et al., 1982). In severe coronavirus infections, cells may fuse to form multinucleate syncytia and/or they may lyse. In contrast to these virulent, cytocidal infections, some tissue culture cells infected with HCV-229E produce virus for weeks or years, without cytopathic effects or cell death (Chaloner-Larsson and Johnson-Lussenburg, 1981a). In cell culture, coronaviruses have a latent period of 6 to 7 hours (Sturman and Takemoto, 1972). Infectious virus can be isolated from culture fluids and from cells disrupted by freezing and thawing (quoted from Holmes, 1985).

Coronavirus replication occurs in the cytoplasm of infected cells. There are limited data on the early events (adsorption, penetration, uncoating, etc.) involved in coronavirus replication. Coronaviruses attach to the cells quickly and equally well at both 4°C and 37°C (Robb and Bond, 1979). Patterson and Macnaughton (1981) have shown that, on monolayer cells infected with HCV-229E, virions are initially attached over the whole cell surface but are then rapidly redistributed away from the cell periphery by an energy-requiring process. It is assumed that upon entering the cell the positive-stranded genome encodes protein(s) whose function is to replicate the genomic RNA and produce subgenomic mRNA (Siddell et al., 1983).

Characteristic of coronavirus infection is the production of 3' coterminal subgenomic RNAs which form a nested set extending in the 5'

direction. These RNAs are capped and polyadenylated. The replicative structures from which they are produced have not been fully characterized, but it has been demonstrated that the negative-stranded template from which murine hepatitis virus mRNAs are copied is of genome length (Siddell et al., 1983). After synthesis, genomic RNA and virion proteins are assembled at the rough endoplasmic reticulum and virions bud into the cisternae, acquiring their lipid membranes from the cell. The virions are subsquently transported to and accumulate in the Golgi complex and smooth-walled vesicles. There is an absence of budding from the plasmalemma. The mechanism of virus release has not been elucidated (Siddell et al., 1983). Human coronavirus 229E replication was shown to be sensitive to actinomycin D (Kennedy and Johnson-Lussenburg, 1979) while other coronaviruses were not (Malluci, 1965).

Coronaviruses primarily infect cells of the respiratory system (HCV, IBV, MHV, and RCV) or the gastrointestinal tract (BCV, CCV, TGEV, TCV, and some MHV strains). Also, many strains of MHV cause hepatitis or encephalomyelitis. These infections are generally acute and it is likely that a lytic infection which destroys the host cell is the basic pathogenetic mechanism involved. Coronaviruses, however, also readily establish persistent infections in animals, often leading to diseases of a subacute or chronic nature (Wege et al., 1983).

Coronaviruses have recently emerged as an important group of animal and human pathogens. Current interest in the molecular biology of coronaviruses centers around the mechanism of the pathogenesis as well as virus-host interactions which lead to subacute or chronic diseases.

ii. Human coronavirus (HCV) infection and its persistence, in vitro.

The first human coronavirus was isolated in 1965 from a patient

suffering from a cold but the virus proved refractory to growth in commonly used types of cell cultures and grew only after several passages in organ culture (Tyrrell and Bynoe, 1965). At about the same time, Hamre and Procknow (1966), working independently in Chicago recovered five virus strains from medical students with colds that grew after one or more blind passages in secondary human embryonic kidney monolayers (quoted from Holmes, 1985). The prototype strain 229E which was eventually adapted to human diploid lung cells (WI-38) was proved by Almeida and Tyrrell (1967) to be identical in its morphology to the strain obtained by Tyrrell and Bynoe (1965). None of the human coronaviruses grows well in cell culture without extensive adaption by passage. A heteroploid human lung cell line, L132, has since been reported to be more reliable for primary isolation of 229E (Bradburne and Tyrrell, 1969), and more recently human fetal tonsil cells (HFT) have been used (Schmidt and Kenny, 1981). Only two human strains, 229E and OC43 have been adapted to cell culture (McIntosh, 1979).

Human coronaviruses (HCV) are responsible for upper respiratory infections, and are associated with lower respiratory tract diseases (reviewed by Wege et al., 1982). Seroepidemiologic studies have shown that HCV infection occurs at all ages and is distributed worldwide. About 15% of common colds are attributed to coronaviruses. In children, pneumonia and other respiratory distress can be caused by coronaviruses. Results of a seroepidemiological survey indicate a possible association of coronaviruses with more severe diseases such as pneumonia, pleurodynia, myocarditis, and meningitis (Riski and Hovi, 1980). In addition to respiratory diseases some human coronaviruses may be associated with enteric infections. However, no information exists on the characteriza-

tion of these agents and their serological relationships to other human coronaviruses (reviewed by Macnaughton and Davies, 1981). Recently, interest has been focussed on human coronavirus because of its possible etiological role in multiple sclerosis (MS) (Burks et al., 1980; Tanaka et al., 1976).

As mentioned previously, coronaviruses of lower animals establish persistent infections in their natural host (Wege et al., 1983). These should provide good in vitro experimental models for the study of chronic disease processes. Coronaviruses also readily establish persistent infections in cell culture (Chaloner-Larsson and Johnson-Lussenburg, 1981a, 1981b; Lucas et al., 1977, 1978; Stohlman et al., 1979; Yoshikura and Tejina, 1981). A persistent infection by human coronavirus 229E (HCV/229E) was established in the human continuous cell line (L132) in our laboratory in 1979. It was the first reported persistent infection of HCV 229E in vitro. The L132 cells persistently infected with and continually shedding high titers of coronavirus have been maintained successfully since their establishment (Chaloner-Larsson and Johnson-Lussenburg, 1981a, 1981b). The persistently infected cells have been termed HV and the virus derived therefrom, VH virus.

The HV cells were regularly passaged at the ratio usually used for normal uninfected cells. Virus titers obtained by plaque assay (Kennedy and Johnson-Lussenburg, 1975/1976) revealed that total virus present in the cultures was consistently 10^5-10^6 PFU/mL. The persistently infected cells were successfully stored at -80°C and revived without difficulty.

The mechanism of the establishment and maintenance of viral persistence in this system has been studied (Chaloner-Larsson and Johnson-Lussenburg, 1981a, 1981b). Defective interfering (DI) particles

and reverse transcriptase could not be detected. The persistently infected cells were resistant to superinfection with standard HCV/229E, but poliovirus replication was entirely unaffected when compared to infection of normal L132 cells. Virulence appeared to be expressed more efficiently in normal cells at 33°C but the persistent virus shedding could only be maintained at 37°C. Therefore, the temperature effect was associated with both the establishment and maintenance of persistence in this system. A recent study has shown that both alpha and beta interferons are involved in the maintenance of this persistent infection (Chudzio and Johnson-Lussenburg, unpublished results). However, unravelling the molecular basis of this persistence in vitro is a major goal for further research.

Our interest lay in the direction of curing the cells of their persistent infection since a comparison of the 'cured' cells with the original uninfected cells, as well as the persistently infected cells, could conceivably contribute to the further understanding of the mechanism involved in the maintenance of persistence in this in vitro system.

2. RIBAVIRIN

Ribavirin, or virazole, or to give it its full name, 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, $C_8H_{12}N_4O_5$, is a synthetic nucleoside which was first synthesized by Witkowski and co-workers in 1972 (Witkowski et al., 1972). It remains, to date, the single most promising broad-spectrum, non-interferon inducing, virustatic agent, effective against both DNA and RNA viruses (Sidwell et al., 1972). This antiviral activity has been demonstrated in cell culture systems, in

organ culture, in animal models, and also in various clinical trials.

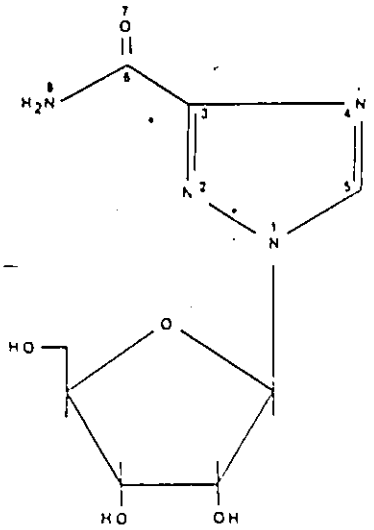
Figure 2 shows the structure of ribavirin. It is a colourless, water-soluble, stable compound. This ribonucleoside analog has a molecular weight of 244, has a ribose moiety, and a triazole ring which resembles guanosine. This structural similarity has been a guiding principle in studies on the mode of action of ribavirin which suggest that guanosine nucleotides may in some way be involved.

i. Mechanism of action of ribavirin.

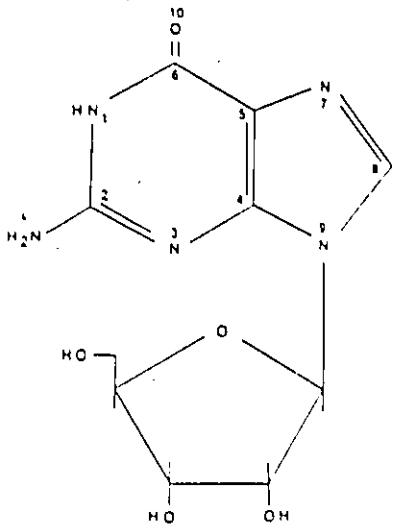
Many studies have been conducted in an attempt to elucidate the biochemical mechanism of the antiviral action of ribavirin, but the precise mechanism involved in its selective antiviral effects against either DNA or RNA viruses is still not clear. Viral attachment and penetration of the host cell are unaffected. The compound is reported to be an inhibitor of both DNA and RNA synthesis, while protein synthesis is apparently only moderately affected following treatment of cells with this drug (Sidwell et al., 1974). In order for ribavirin to exert its antiviral action, it must first be phosphorylated (Sidwell et al., 1979; Streeter et al., 1973). The compound is converted to its 5'-mono-, di-, and triphosphates by cellular enzymes (Miller et al., 1977; Sidwell et al., 1979; Streeter et al., 1974), and data reported by both Smith et al. (1974) and Streeter et al. (1973) indicate that ribavirin 5'-monophosphate inhibits the synthesis of guanosine 5'-monophosphate (GMP) by blocking the conversion of inosine 5'-monophosphate (IMP) to xanthosine 5'-monophosphate (XMP), a reaction catalyzed by IMP dehydrogenase (Fig 3). As a result of this inhibition, the supply of dGTP is curtailed with accompanying depletion of both the GTP and dGTP pools, resulting in the inhibition of nucleic acid synthesis (Muller et al., 1977). In addition,

FIGURE 2

Figure 2. Structure of ribavirin and its resemblance to guanosine.



ribavirin



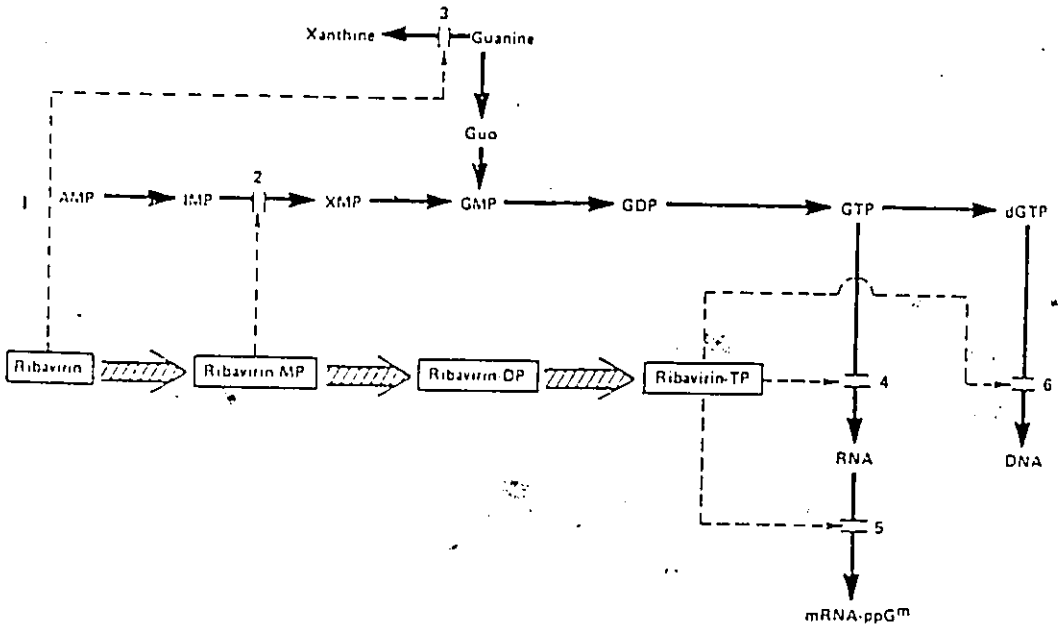
guanosine

FIGURE 3

Figure 3. Metabolism and possible biochemical targets for the antiviral actions of ribavirin.

The large arrows indicate the metabolic pathways; the small broken arrows indicate the known effects of ribavirin and its metabolites on specific enzyme systems. 1: deoxyadenosine. Enzymes involved: 2: IMP dehydrogenase, 3: guanine deaminase, 4: influenza virus-associated RNA polymerase, 5: vaccinia virus-associated GTP-mRNA guanylyltransferase, and 6: herpesvirus-induced DNA polymerase.

Taken from Shannon, W.M. (1984).


3
Xanthine
Guanine
Guo
1
AMP
IMP
2
XMP
GMP
GDP
GTP
dGTP
Ribavirin
Ribavirin MP
Ribavirin DP
Ribavirin-TP
4
6
RNA
DNA
5
mRNA-ppG^{m}

the enzyme guanine deaminase is inhibited by ribavirin (Muller, 1979).

Oxford (1975, 1976) has reported that ribavirin selectively inhibits influenza virus replication and the synthesis of virus-specific polypeptides at concentrations that do not affect cellular protein synthesis.

Ribavirin 5'-triphosphate has also been found to inhibit vaccinia virus-associated mRNA guanylyltransferase activity, thereby blocking the capping of virus-specific mRNA (Goswami et al., 1979). This finding could possibly provide an explanation for the antiviral activity of ribavirin against both DNA and RNA viruses, and for its marginal effectiveness against poliovirus, which does not require a capped mRNA.

Herpes simplex virus DNA synthesis is selectively blocked when compared with cellular DNA synthesis in virus-infected cells (Drach et al., 1981). The selective inhibition of herpes virus DNA synthesis may be partially due to the inhibition of the virus-induced DNA polymerase by ribavirin 5'-triphosphate (Oberg and Helgstrand, 1978).

ii. <u>In vitro antiviral activity</u>.

Extensive studies on the in vitro antiviral activity of ribavirin have been reported and their results are summarized in Tables 2 and 3. It is obvious that the antiviral spectrum of this triazole nucleoside is indeed wide. Virtually all viruses have a degree of sensitivity to the drug. The extent of activity has varied considerably in relation to the virus tested, cell line utilized and parameter for measuring the antiviral activity.

DNA virus-inhibitory effects (Table 2):

Members of the Herpesviridae family all appear quite sensitive to ribavirin, with the exception of pseudorabies virus which is inhibited

Table 2. Anti DNA virus activity of ribavirin in cell culture systems

Virus	Cell culture*	Antiviral activity**
Adeno 2	HEp-2	-
Adeno 3	KB	+
Adeno 5	HeLa	+
Adeno 5	KB	-
Adeno 19	HeLa	+
Herpes 1	KB, RK-13, CE, HeLa	+
	RK, HSF	+
	Vero, WI-38	-
Herpes 2	KB, RK-13, HSF	+
	CE, Vero	-
Turkey herpes	CE	+
Mareks disease	CE	+
Varicella zoster	WI-38	-
Human cytomegalo	WI-38	+
Murine cytomegalo	ME	+
Pseudorabies	RK-13, Vero	-
Infectious bovine rhinotracheitis	BET	+
Feline rhino-tracheitis	NLFK	+
Vaccinia	KB, CE, HeLa	+
	Vero	+/-
	RK,BSC-1	+
Myxoma	RK-13	+

* BET.....Bovine embryonic trachea
CE.....Primary chick embryo
HEp-2....Human epidermoid carcinoma of the larynx
KB,.....Human carcinoma of the nasopharynx
RK.....Primary rabbit kidney
Vero.....African green monkey kidney
BSC.....Green monkey kidney
HeLa.....Human carcinoma of the cervix
HSF.....Human skin fibroblast
ME.....Mouse embryo
NLFK.....Feline kidney
RK-13.....Rabbit kidney
WI-38.....Human embryonic lung fibroblast

** + Positive activity as defined by the investigator performing the study;
- Negative activity , also as defined by the investigator.

Adapted from Sidwell (1980).

only at high concentrations. Against human herpesvirus type 1 and 2, ribavirin's minimum inhibitory concentrations (MIC) have ranged from 0.32 to 32 ug/mL or greater, depending on the virus concentration and cell line used (Descamps and De Clercq, 1978; Huffman et al., 1973; Sidwell et al., 1972). The human and murine cytomegaloviruses appeared somewhat less sensitive, with MIC's of 10 to 32 ug/mL (Dowling et al., 1976; Huffman et al., 1973; Sidwell et al., 1972, 1974).

Adenoviruses are quite insensitive to the effects of most antiviral compounds (Sidwell and Witkowski, 1979). However, most of the adenovirus types evaluated were inhibited by ribavirin. The MIC's generally varied from 10 to 200 ug/mL, depending on the virus studied and parameter used for evaluation (Huffman et al., 1973; Scheffler et al., 1975; Sidwell et al., 1972).

Vaccinia and myxoma viruses have appeared to be inhibited by ribavirin (Descamps and De Clercq, 1978; Huffman et al., 1973; Katz et al., 1976; Odelola, 1978; Sidwell et al., 1972). In the presence of ribavirin, viral DNA and viral polypeptides were formed in the cells, but the polypeptides failed to coat the virus DNA to form intact virions (Katz et al., 1976).

RNA virus-inhibitory effects (Table 3):

Influenza viruses are inhibited by ribavirin in vitro. To date, at least 28 strains of influenza A virus have been found sensitive to the drug, with MIC's reproducibly seen as low as 0.05 ug/mL, as reported by Togo (1973) measured by virus titre. Importantly, influenza B virus seems as sensitive to the drug as influenza A virus (Huffman et al., 1973; Sidwell et al., 1972; Togo, 1973).

Viruses of the Paramyxoviridae family, including parainfluenza 1

Table 3. Anti RNA virus activity of ribavirin in cell culture systems

Virus	Cell culture*	Antiviral activity**
Influenza A	CE, CC, BSC-1, CK, HeLa, RMK	+
Influenza B	CE, MK	+
Parainfluenza 1	CE. KB, Vero, CK	+
	BSC-1	+/-
Parainfluenza 2	CK	+
Parainfluenza 3	KB	+
Measles	Vero	+
Subacute sclerosing panencephalitis	Vero	+
Mumps	BHK	+
Newcastle disease	CE	+
Rhino 1A, 2, 8, 13, 56	KB	+
Rhino 2, 42	HEL, WI-38	-
Rhino 1	MK, WI-38	+
Corona 229E	WI-38	+
Corona 229E	HEL	-
Reo 1, 2, 3	BK	+
Simian rota	?	+
Calf, porcine rota	MK	+
Coxsackie A21	HeLa, HEL, WI-38	+
Coxsackie B	RK, HeLa	+
	Vero	+/-
Polio 1, 2	HeLa	+
Polio 2	KB	+/-
Sindbis	BHK	+
	CE	+/-
Semliki Forest Virus	CE, L929	+
	WI-38	-
St. Louis Encephalitis	BHK	+
Chikungunya	Vero	+
Simian foamy 1, 2, 4	BHK, HeLa	+
Vesicular stomatitis	KB, HEL	+
Chandipura	Vero	+
Moloney sarcoma	?	+
Bovine leukemia	FLK, F-81	+
Gross leukemia	XC	+

* BHK.....baby hamster kidney
CC.....Chang's conjunctival
FLK.....Fetal lamb kidney
L929.....Mouse fibroblast
XC.....Rat sarcoma
BK.....Bovine kidney
CK.....Calf kidney
HEL.....Human embryonic lung
MK.....Rhesus monkey kidney
See other definitions under footnote Table 2.

** See footnote Table 2.

Adapted from Sidwell (1980).

and 3, mumps, respiratory syncytial, measles, subacute sclerosing panencephalitis (SSPE)-derived measles strains and Newcastle disease viruses have all proven to be markedly susceptible to ribavirin in vitro (Descamps and De Clercq, 1978; Hruska et al., 1980; Huffman et al., 1973; McCammon and Riesser, 1979; Murphy, 1978; Sidwell et al., 1972, 1975; Streeter et al., 1973). Measles virus has appeared to be the most sensitive of this group of viruses, with MIC's as low as 0.0032 ug/mL. McCammon and Riesser (1979) have shown that ribavirin had marked antiviral activity against a persistent infection of mumps virus grown in BHK-21 cells.

The picornaviruses, such as polio, coxsackie and rhinoviruses are probably least affected by ribavirin in cell culture systems (Huffman et al., 1973).

Unpublished observations have been reported that coronavirus 229E is inhibited by ribavirin in WI-38 cells but not in HEL cells (Sidwell et al., 1974).

Several viruses in the Reoviridae family are relatively sensitive in vitro to ribavirin. Viruses inhibited include reo 1,2 and 3, bovine rota, simian rota, and porcine rota, with MIC's ranging from 0.32 to 10 ug/mL (Sidwell, 1980).

Among the viruses of the Togaviridae family inhibited by ribavirin are Sindbis, Chikungunya, Semliki Forest (Alphaviruses), and St. Louis encephalitis (Flavivirus). These viruses would be considered of moderate sensitivity, with MIC's usually ranging from 1.5 to 32 ug/mL (Descamps and De Clercq, 1978; Huffman et al., 1973; Sarver and Stollar, 1978).

Vesicular stomatitis virus and Chandipura virus are inhibited by ribavirin, the MIC's ranging from 1.5 to 320 ug/mL (Huffman et al.,

1973; Odelola, 1978; Sidwell et al., 1972).

Ribavirin has exhibited significant in vitro activity against Moloney sarcoma, Gross leukemia, and bovine leukemia viruses (Shannon, 1977). The simian foamy viruses, a type B oncovirus or spumavirus, are also reported sensitive to ribavirin (Sidwell, 1980). More recently, McCormick et al. (1984) reported that the replication of human T-lymphotropic virus, type III (HTLV-III), in human adult T-lymphocytes was suppressed by ribavirin.

Cytotoxicity of ribavirin:

Ribavirin's cytotoxic effects have not varied substantially in the cell lines studied. The 50 percent cytotoxic dose ($CCED_{50}$) in the resting cell is approximately 200-1000 ug/mL of ribavirin, as determined by microscopic examination of cell monolayers, vital staining, plating efficiency of viable cells, and total cellular protein (Huffman et al., 1973). The compound is considered quite cytostatic on cells, preventing cell division as long as the drug remains in contact (Huffman et al., 1973; Muller et al., 1977). This cytostatic effect, as measured in mouse lymphoma cells, is readily reversed by guanosine, xanthosine and inosine (Muller et al., 1977). Ribavirin does not appear to be incorporated into cellular materials.

In vitro studies have not been able to demonstrate ribavirin-resistant strains of herpes or parainfluenza viruses (Allen and Fingal, 1977; Huffman et al., 1977; Sidwell et al, 1979).

iii. In vivo efficacy of ribavirin.

Of the DNA viruses explored, the best activity has been seen in herpes and poxvirus families (Allen et al., 1977; De Clercq et al., 1976). Some activity has been observed with a papovavirus infection

(Sidwell et al., 1975). Intraperitoneal and topical routes were found to be effective in reducing herpesvirus-induced lesions (Allen et al., 1977; Sidwell et al., 1973), and preventing encephalitic deaths (Allen et al., 1977; De Clereq and Luczak, 1976). This may suggest that drug treatment sufficiently controlled virus replication at the initial site to prevent the neuronal transfer of the virus to the brain. Therefore, it might be possible for ribavirin to prevent latency if treatment were initiated very early in a primary infection.

Of the RNA viruses studied in vivo, good activity had been seen with 6 of the 10 families: arena-, bunya-, corona-, orthomyxo-, paramyxo-, and retroviruses. Contradictory, marginal, or negative findings were observed with picorna-, rhabdo-, reo-, and togaviruses (Allen, 1980). Impressive and promising findings have been observed in monkeys which were infected with Lassa fever or Machupo (arenavirus) and then treated intramuscularly with ribavirin. Similar results occurred following ribavirin treatment of Pichinde infected hamsters. In a limited trial, an effect also has been shown against Hantaan virus (bunyavirus) in the striped field mouse, the only animal model for Korean haemorrhagic fever. Also activity has been seen with another member of the same family i.e. Rift Valley fever virus (Allen, 1980).

However, interpolation of these in vivo data to man is practically impossible because of the differences between man and animals and the many factors affecting the results obtained in studies with laboratory animals (Table 4). Of importance among these factors are basic species differences which may be reflected in variations of drug metabolism, and pathogenesis affecting severity. Practical human usage will require definition of optimal vehicles, administration dosages and schedules in

Table 4. Factors affecting in vivo antiviral test systems

- **A. Agent**
  1. virulence
  2. size of inoculum
  3. route of administration
  4. degree of adaptation to host
- **B. Host**
  1. species
  2. age
  3. sex
  4. weight
  5. general health
  6. supply of food and water
  7. supportive care
  8. stress
     a. capture
     b. treatment
- **C. Drug**
  1. absorption
  2. metabolism
  3. distribution
  4. exertion
  5. toxicity
  6. formulation
  7. route of administration
  8. schedule of administration
  9. reversing agent availability
- **D. Other**
  1. secondary infections

Taken from Allen (1980).

various human infections.

In experimental animals, the drug does produce a macrocytic anemia which is characterized by a progressive reduction in erythrocytes and blood hemoglobin concentrations. Ribavirin does not alter the fertility or reproductive function of either male or female rats, and no evidence of mutagenic toxicity was detected in several tests in vivo and in vitro. The drug was found, however, to be significantly embryolethal in rats and rabbits, and teratogenic in rats but not baboons (Hillyard, 1980). The exact mechanism by which ribavirin produces those toxic changes observed in animals has not been completely clarified. Whatever the toxic mechanism of action may be, with the exception of embryonic effects, it appears not to be due to a permanent or irreversible change. All adverse effects produced by ribavirin reverse readily when drug administration is stopped.

Ribavirin has biological activities in addition to its antiviral and cytotoxic effects. Some reports have shown that ribavirin does not reduce the production of interferon nor did the treatment increase the susceptibility of mice to influenza virus. An important observation was that the influenza virus-infected mouse, treated with ribavirin, was still able to be sufficiently immunized to overcome subsequent viral challenge (Jolley et al., 1980). However, it would appear that ribavirin has definite immunoregulatory functions. At lower concentrations, it appears to stimulate immune responses. When the concentration of the drug is increased, it appears that a definite inhibition of the immune response occurs depending upon the time the agent is administered relative to antigenic challenge, the dose used, and the animal model used (Jolley and Suchil, 1984). In experimental animals, ribavirin has thera-

peutic activity against transplantable virus-induced tumors (Jolley et al., 1977; Newman et al., 1977; Potter et al., 1976) and autoimmune diseases (Klassen et al., 1979).

However, regardless of the cause and mechanism, the overall toxicity profile of ribavirin in laboratory animals is significantly suggestive of safety in man.

iv. Clinical trials with ribavirin.

Most effective antiviral therapies currently available are directed at the herpes group of viruses, and the important RNA viruses have not yielded to safe treatment with an antiviral agent. It is in this area of serious viral infections caused by RNA viruses that ribavirin most clearly merits the designation as promising.

To date, the best success has occurred in the use of ribavirin to treat respiratory syncytial virus (RSV) infection in infants and young children. In a randomized, double blind trial, ribavirin was administered by continuous aerosol for three to six days to infants who were hospitalized with proven respiratory syncytial virus infection. Ribavirin treatment reduced severity of illness by day 3, as well as virus shedding and viral titre. The virus did not become resistant to the drug. No toxic side effects were noted in any infant (Hall et al., 1983).

The course of both influenza A and influenza B infections in adults was favorably affected by ribavirin therapy, especially when the agent is administered as a small particle aerosol. In randomized controlled studies, ribavirin aerosol was effective in the treatment of influenza A (H1N1) during an outbreak among college students and in the treatment of type B influenza virus infection (Knight et al., 1981; McClung et al.,

1983). The treatment reduced clinical manifestations as well as virus shedding.

Success has also been reported in the use of ribavirin to treat severe and life-threatening infections with Lassa fever virus. An important study by McCormick et al. (1986) has shown that intravenous or oral therapy with ribavirin is associated with a dramatic clinical improvement and reversal of severe symptoms. This represents a significant advance in antiviral therapy.

The effectiveness of oral ribavirin against measles has been reported in Mexico, Brazil, and the Philippines (Banks and Fernandez, 1984). In three double-blind, placebo-controlled studies, ribavirin reduced both the severity and duration of the clinical manifestations of the disease, as well as the complications usually associated with this viral infection.

Favorable results with oral ribavirin treatment of acute hepatitis A virus infection have been reported (Sanchez et al., 1984). There was more rapid recovery from illness and return to normal liver function in treated patients.

Ribavirin treatment has produced encouraging results in patients with herpes virus infections, including herpes zoster, herpes genitalis infections, and herpetic gingivostomatitis. The drug reduced the duration of lesions and of pain (Fernandez and Diaz-Perches, 1977), and also decreased the duration of lesion-healing.

Although ribavirin was very well tolerated in many clinical studies, reported side effects have included transient elevations in bilirubin, fall in hematocrit, shortened red cell survival, and a possible drug induced hemolytic anemia (Canonico et al., 1984;

Fernandez, 1980).

The data from a large number of clinical trials indicate that relatively safe oral doses of ribavirin could approach 3,300 mg/day for seven days in adult patients. At high doses (3,900 to 12,600 mg/day) a decline of hemoglobin may be seen by day 7 to day 13 of treatment. It is however, rapidly reversible upon withdrawal of the drug and/or when transfusions are administered to correct the anemia (Fernandez, 1980).

Ribavirin was found to be teratogenic and embryotoxic in rodents but not primates. However, these results cannot be extrapolated and until further experience is available, ribavirin should not be administered during human pregnancy and the lactation period. No neurologic toxicity has been observed. No resistance to ribavirin has been demonstrated in clinical use (Fernandez, 1980).

However, ribavirin appears to be reasonably nontoxic, to have an acceptable metabolic disposition, and its production is remarkably low in cost (Sidwell et al., 1974), although it might be expensive in clinical use. The drug seems to be well suited for formulation as a clinical drug. Ribavirin is now commercially available in many countries around the world for the treatment of viral diseases. It has been recently approved in the United States and Canada for the treatment of severe cases of RSV in infants and young children, and there are several on-going clinical trials. The biochemistry and clinical applications of ribavirin were recently reviewed by Gilbert and Knight (1986).

PURPOSE OF RESEARCH

It is well recognized that coronaviruses are an important group of viruses that are responsible for a variety of diseases in man and animals, ranging from inapparent infections to acute and chronic disorders. Seroepidemiologic surveys and virus-isolation studies indicate that human coronaviruses cause about 15-20% of upper respiratory infections in humans, and that they may play a role in other human diseases such as pneumonia and myocarditis. Since there are no immunological means to control HCV infection at present, development of an effective antiviral agent could help in ameliorating morbidity.

Ribavirin is a synthetic nucleoside which has antiviral properties against a large number of both ribonucleic acid and deoxyribonucleic acid viruses in vitro and in vivo. Unpublished observations have shown that HCV/229E is inhibited by ribavirin in WI-38 cells (Sidwell, 1980). However, we have found no published report of the effect of ribavirin on HCV infection, especially on HCV persistent infection in vitro. We were, therefore, interested to determine the potential of ribavirin on HCV acute and persistent infections in vitro, and more specifically, to attempt to cure the HCV persistent infection which has been maintained in our laboratory since its establishment in 1979.

The purpose of this study was to:

i) establish the effect of ribavirin on host cell replication;

ii) investigate the antiviral activity of ribavirin on HCV acute and persistent infections in vitro;

iii) evaluate the effect of ribavirin on HCV plaque formation; and

iv) attempt to cure the HV cells of their persistent infection by continuous passage of the cells in the presence of ribavirin.

II. MATERIALS AND METHODS

1. CELLS AND CELL CULTURES.

i. Human embryonic lung cells L132, a continuous cell line, were used throughout this study for virus production and plaque assay according to procedures previously described (Kennedy and Johnson-Lussenburg, 1975/1976). The cells were grown in Eagle's minimal essential medium (MEM; Flow Laboratories Inc., Mississauga, Ontario, Canada) supplemented with 10 percent fetal bovine serum (FBS), sodium bicarbonate (20 mM), penicillin (100 units/mL), streptomycin (100 ug/mL), neomycin (50 ug/mL) and glutamine (2 mM), and incubated at 37°C in a 5% CO_2 atmosphere.

ii. L132 cells persistently infected with human coronavirus 229E termed HV cells have been maintained in this laboratory since 1979 (Chaloner-Larsson and Johnson-Lussenburg, 1981a, 1981b). The cells were passaged regularly at 1:3 split ratios every 2-3 days, and incubated at 37°C in a 5% CO_2 atmosphere. Disposable cell culture flasks (80 cm^2, Gibco Canada Inc.) were used for cell passage and preparation of virus inoculum, and 12 flat-bottomed, disposable tissue culture plates (Linbro, Flow Labs. Inc. Mclean, VA.) were used for the antiviral experiments.

iii. Growth of cells in the presence of ribavirin.

2.0 mL/well of L132 or HV cells, suspended in MEM with supplements, were seeded into 12-well plates at concentrations of approximately $2.0x10^5$ cells/mL. Medium was removed 24 h after seeding and replaced with fresh MEM containing ribavirin at 0, 10, 50, 100, and 500 ug/mL. According to the experimental design, the effect of each ribavirin concentration was tested in triplicate at 24, 48, and 72 h post

treatment. At these times, cell monolayers were evaluated microscopically for cytotoxic effects such as cell granulation, alteration in cell shape, presence of detached and floating cells, etc. Then the supernatant fluids (SNFs) from three wells at each test concentration relevant to the time period were collected and the underlying cell monolayers washed 3X with phosphate-buffered saline (PBS) and trypsinized with 0.2 mL trypsin. Equal volumes of PBS were added to each well, the released cells from the 3 wells were pooled. Viable cell counts of both SNF and cell samples were made by erythrosin B exclusion. In this procedure, equal volumes (25 uL) of 0.4% erythrosin B solution were added to equal volumes of cell samples. Viable cells excluding the dye and pink, non-viable cells were counted in a haemocytometer.

2. VIRUSES AND VIRUS PRODUCTION.

i. Human coronavirus strain 229E (HCV/229E) was originally obtained from Dr. A. Z. Kapikian at the National Institutes of Health, Bethesda, MD. It was reported that it had been passed in WI-38 cells. After an initial two passages in WI-38 cells in our laboratory it has been cultivated only in L132 cells.

ii. VH virus is the virus derived from HCV/229E persistently infected HV cells. The HV cells were passaged regularly at a 1:3 split ratio every 2-3 days, and served as the source of VH virus when required.

iii. Preparation of virus inoculum. HCV/229E and persistent VH viruses were propagated as acute infections in L132 cells. Monolayers of L132 cells in flasks, were inoculated with virus at an approximate multiplicity of infection (MOI) of 0.01 to 1.0, adsorbed at room

temperature for 1 h, then fed with M199 without FBS, and incubated at 33°C for 36-40 h. The monolayers were frozen and thawed 3 times to release the cells and virus, the suspensions were pooled, dispensed into small vials, and were stored at -80°C. Titers of virus pools were determined by the plaque assay described below.

3. CHEMICAL AGENTS.

i. Ribavirin (Virazole) was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio. A working stock of ribavirin (1 mg/mL in MEM) was prepared and sterilized by passage through a 0.2 um membrane filter (Nalge Company, Rochester, N.Y.). The stock solution was dispensed into small vials and stored at -20°C until needed. The appropriate concentrations of the drug were made by either dilution of the stock solution in cell culture medium (MEM) or incorporated into the agar overlay. The formula of agar overlay is provided in the Appendix.

ii. Guanosine was purchased from ICN National Biochemicals, Cleveland, Ohio. For some experiments a solution of guanosine (200 ug/mL) was made by dissolving required amounts in MEM, and sterilized by passage through a 0.2 um membrane filter. Test concentrations were made by further dilution of this solution in MEM. For plaquing tests, a working stock of guanosine (500 ug/mL) was made by dissolving the compound in distilled water, and autoclaving. Appropriate amounts of 500 ug/mL guanosine were added to melted 1.8% agar held at 42°C to obtain a final concentration of 200 ug/mL guanosine in the 0.6% agar overlay (details in Appendix).

4. PLAQUE ASSAY.

Stock inocula of both 229E and VH viruses were titrated by plaque assay in monolayers of L132 cell grown in 80 cm^2 flasks following the procedures described previously (Kennedy and Johnson-Lussenburg, 1975/1976). For plaque development, 229E incubation was at 33°C for 6 days, and VH, 5 days. Virus titers were expressed as plaque-forming units per mL (PFU/mL).

Experimental samples of both SNFs and cells were plaque-assayed in triplicate in L132 cells grown in 12-well plates following the same procedures as described above. The plates, however, as is the practice when using flasks, could not be inverted during incubation. Results have consistently shown that there is approximately a 1 log difference in the titers; those obtained in 12-well plates always being lower.

5. DETERMINATION OF ANTIVIRAL ACTIVITY OF RIBAVIRIN.

i. Ribavirin effect on HCV production: In HCV acute infection, 0.2 mL per well of either 229E or VH virus was inoculated onto L132 cell monolayers grown in 12-well plates at a multiplicity of infection (MOI) of approximately 1-2.5 for 229E and 15-60 for VH virus. The viruses were allowed to adsorb at room temperature for 1 h prior to the addition of 2 mL of MEM containing appropriate amounts of ribavirin (10-100 ug/mL) to each of three wells used to test each drug concentration. The cells were then incubated at 33°C for 48 h. After evaluating the monolayers, the supernatant fluids at each of the different concentrations were collected; if present, detached cells in the SNF were counted to monitor for cytotoxicity of the drug. The virus released into the SNF was plaque assayed. The cell monolayers were washed 3x with PBS, trypsinized, and

suspended in PBS (final vol. 0.4 mL/well). Total viable cells were counted and samples were assayed for cell bound virus. Both SNF and cell samples were stored at -80°C until assayed. All samples were subjected to 3 freeze-thaw cycles before assay.

To evaluate the effect of ribavirin on HCV persistent infection, the supernatant fluid (MEM) of 24 h HV cell monolayers grown in 12-well plates were replaced in triplicate with MEM containing test concentrations of ribavirin. Incubation was contidued at 15/C for 48 h. The supernatants and cells were then collected as described, total and viable cells were counted, and the released and cell bound virus was assayed.

ii. Inhibition of 229E and VH virus plaque formation by ribavirin.

Serial 10-fold dilutions of either 229E or VH viruses were inoculated onto L132 cell cultures grown in 12-well plates. After a 1 h adsorption period, the monolayers were covered by the agar overlay containing ribavirin at the test concentrations (1, 10, 20, 30, 50 and 70 ug/mL). Controls were done in parallel at each virus dilution and contained no ribavirin. All tests were performed in duplicate. The plates were incubated at 33°C for 5-6 days to develop plaques. The results were expressed as % inhibition of control PFU/mL obtained at each dilution of input virus.

iii. Procedure for curing HV persistently infected cells with ribavirin.

After several unsuccessful attempts to maintain the HV persistently infected cells in the presence of ribavirin, the following procedure was adopted. Three flasks of HV confluent cell monolayers were rinsed with PBS, and refed with MEM containing 10, and 50 ug/mL ribavirin, respect-

ively. Control cultures were carried in parallel but without ribavirin. After incubation at 37°C for 24 h, the medium was removed and tested for infectivity. The cells were passaged following standard procedures and maintenance under the same concentrations of ribavirin was continued. Further subcultures were made at intervals of 2 or 3 days depending on the condition of the cells, still under the same drug concentrations. Samples of both supernatant and cell fractions at each cell passage were collected, cells were counted, and plaque assays were carried out on every cell passage. For further evaluation, all the SNFs from the monolayers at each passage were pooled and stored at -80°C.

6. DETERMINATION OF THE EFFECT OF GUANOSINE ON THE ANTIVIRAL ACTIVITY OF RIBAVIRIN.

i. Effect of guanosine on the inhibition of plaque formation by ribavirin.

The following procedures using guanosine were modified from Streeter et al. (1973). L132 cells, grown to confluency in the 12-well culture plates were infected with 10-fold dilutions of either 229E or VH virus. Duplicate wells were used for each viral dilution (0.2 mL/well). After adsorption of the viruses at room temperature for 1 hour, 2 mL of an agar overlay containing either no additive, 50 ug/mL of ribavirin with or without 200 ug/mL guanosine or 200 ug/mL guanosine alone was added to each well. Preparation of ribavirin and guanosine stock solutions has been described above. The plates were incubated at 33°C for 5-6 days to develop plaques.

ii. Effect of guanosine on the inhibition of viral repliction by ribavirin.

To determine whether guanosine could block the antiviral effect of

ribavirin during 229E and VH virus production acute infection, the following experiments were performed. Monolayers of L132 cells grown in 12-well plates were infected with either 229E (MOI 0.2-0.7) or VH (MOI 15-30) virus. After 1 h viral adsorption at room temperature, the monolayers were refed with MEM containing either no additive, 50 ug/mL of ribavirin with and without 200 ug/mL guanosine or 200 ug/mL guanosine alone and incubated at 33°C for 48 h. SNFs were then collected, cells were washed, trypsinized and counted, and samples were plaque assayed.

The effect of guanosine on the antiviral effect of ribavirin in HV persistently infected cells was tested using the following experimental protocol. 24 h HV cell monolayers were refed with MEM containing either no additive, 50 ug/mL ribavirin with and without 200 ug/mL guanosine, and guanosine (200 ug/mL) alone. After incubation at 37°C for 48 h, SNFs and cell fractions were harvested and assayed as previously described.

7. STATISTICAL METHODS.

Computer analyses were performed through the courtesy of Dr.R.C. Nair, Department of Epidemiology and Community Medicine, University of Ottawa. BMDP, a statistical software program was used for the analysis of variance and covariance with repeated measures (BMDP 2V) to evaluate the experimental results. Polynomial (quadratic) regression (BMDP 5R) was also used to evaluate the effect of ribavirin on the HCV acute and persistent infections and to draw the regression curves presented in Fig. 8.

III. EXPERIMENTAL RESULTS

1. EFFECT OF RIBAVIRIN ON HOST CELL REPLICATION.

Before testing the antiviral properties of ribavirin, the growth of both L132 and HV cells was tested in the presence of several ribavirin concentrations to determine whether the drug had any effect on the replication of the host cells. Even in the presence of 500 ug/mL of ribavirin, no significant changes in the morphology of the cells in monolayers of either cell type were observed over the 72 h test period. The cell numbers of both types were determined at each test period (24, 48 and 72 h) of treatment with different concentrations of ribavirin. The results of the total cell counts for both cell types were expressed as percent inhibition of control cells grown in parallel in the absence of ribavirin and are shown in Fig. 4. The numbers of viable cells within each population were determined and are shown in Tables 5 and 6.

These results clearly demonstrate that, with the higher concentrations of ribavirin (100-500 ug/mL), there was a progressive reduction in both total and viable cell numbers for both L132 and persistently infected HV cells after 24, 48 and 72 h treatment. However, during the first 24 h, the effect of the lower concentrations (10 & 50 ug/mL) was less pronounced. There were wide fluctuations in cell numbers and in some cases cell replication seemed to be enhanced, especially the L132 cells treated with 10 ug/mL. From these results, it appeared that the effect of ribavirin was associated more with increasing concentration (dose/response) than with the period of incubation (time/response). It was also seen that the effect of ribavirin on the HV cells was similar to that observed on L132 cells; however, there was overall a greater

FIGURE 4

Figure 4. The % inhibition of total cells/mL by different concentrations of ribavirin on (A) L132, and (B) HV cells at 24, 48 and 72 h post-treatment at 37°C.

I: mean of 3 experiments ± S.D.

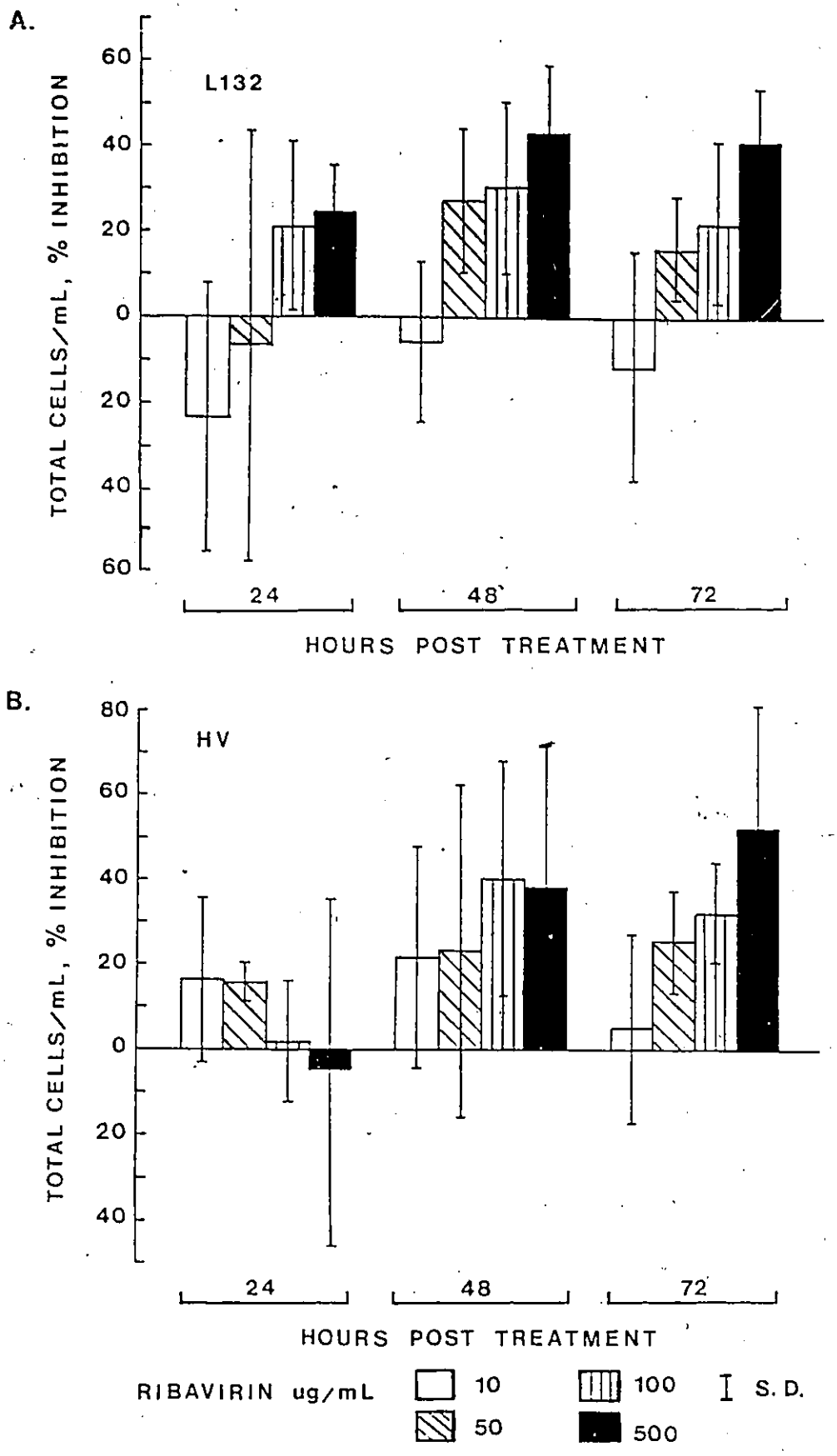

A.
L132
TOTAL CELLS/mL, % INHIBITION
60
40
20
0
20
40
60
24
48
72
HOURS POST TREATMENT
B.
HV
TOTAL CELLS/mL, % INHIBITION
80
60
40
20
0
20
40
24
48
72
HOURS POST TREATMENT
RIBAVIRIN ug/mL
10
50
100
500
S.D.

Table 5. The effect of ribavirin on the replication of L132 cells at 37°C.

RIBAVIRIN	NUMBER OF VIABLE CELLS X 10^5/mL*		
ug/mL	24 h	48 h	72 h
Control	24.8±8.3	31.1±2.7	28.1±8.6
10	29.4±8.9	33.9±7.0	32.2±14.4
50	23.2±1.9	21.4±5.5	23.0±4.6
100	20.2±10.0	21.5±6.6	21.1±3.3
500	18.2±9.2	17.3±4.9	15.4±2.0

* Mean of 3 experiments ± S.D.

Table 6. The effect of ribavirin on the replication of HV persistently infected cells at 37°C.

RIBAVIRIN	NUMBER OF VIABLE CELLS X 10^5/mL*		
ug/mL	24 h	48 h	72 h
Control	17.9±10.9	25.1±13.9	27.8±18.4
10	17.1±2.8	16.3±4.3	23.4±11.5
50	15.2±8.0	15.9±6.5	19.8±9.9
100	18.2±6.3	12.5±5.8	18.0±10.3
500	19.4±7.2	11.3±4.5	11.4±4.5

* Mean of 3 experiments ± S.D.

variation in cell numbers. On the basis of these results, it was concluded that, although the effect on HV cells was more variable, ribavirin exerted a marked cytostatic effect on both host cell types. Analysis of variance and covariance with repeated measures was used to evaluate the relationship between different concentrations of ribavirin and numbers of total cells/mL post treatment. The results confirmed that the differences among concentrations were statistically highly significant for the L132 cells ($p = 0.0006$) but of marginal significance for the HV cells ($p = 0.19$).

The effect of ribavirin treatment on the viability of L132 and HV cells was evaluated by analyzing the percentage viability of the cells over the duration of the experiments. There was no significant reduction in L132 cell viability which ranged between 87.8-97.0% (Table 7) at the different times of treatment and different concentrations of ribavirin (dose/response $p = 0.39$) . The results for the persistently infected HV cells (Table 8) were similar and although the percent viability was lower and fluctuated more widely (78.2-90.2%), the differences were not significant (dose/response $p = 0.59$). Therefore, it was concluded that ribavirin was not toxic to either the L132 or HV cells.

A comparison of the effect of different concentrations of ribavirin on both cell types after incubation at 37°C for 72 hours was made by plotting the percent inhibition of total cells. The results are shown in Figure 5 where the relationship between the concentrations of ribavirin and the inhibition of total cells indicate that the 50% inhibition of cell replication occurred at ribavirin concentrations equal to or greater than 100 ug/mL for both cell types. Also, the HV persistently infected cells seemed to be more sensitive to the effect of ribavirin.

Table 7. The effect of ribavirin on the viability of L132 cells at 37°C.

RIBAVIRIN	% VIABLE CELLS*		
ug/mL	24 h	48 h	72 h
Control	97.0±1.7	91.7±6.8	92.1±2.0
10	96.6±0.1	94.3±3.3	92.0±1.2
50	95.2±1.6	87.8±7.3	92.9±3.9
100	96.4±1.1	91.8±2.9	93.4±0.8
500	89.7±10.3	90.9±3.7	91.0±4.1

* Mean of 3 experiments ± S.D.

Table 8. The effect of ribavirin on the viability of HV persistently infected cells at 37°C.

RIBAVIRIN	% VIABLE CELLS*		
ug/mL	24 h	48 h	72 h
Control	78.4±14.2	89.2±4.2	80.6±9.2
10	82.2±12.2	86.2±4.0	78.2±8.3
50	90.2±6.0	86.7±7.2	82.9±4.6
100	87.6±5.6	85.9±5.7	78.9±7.7
500	89.4±4.2	78.2±15.1	79.2±10.4

* Mean of 3 experiments ± S.D.

FIGURE 5

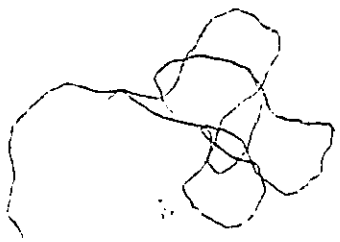
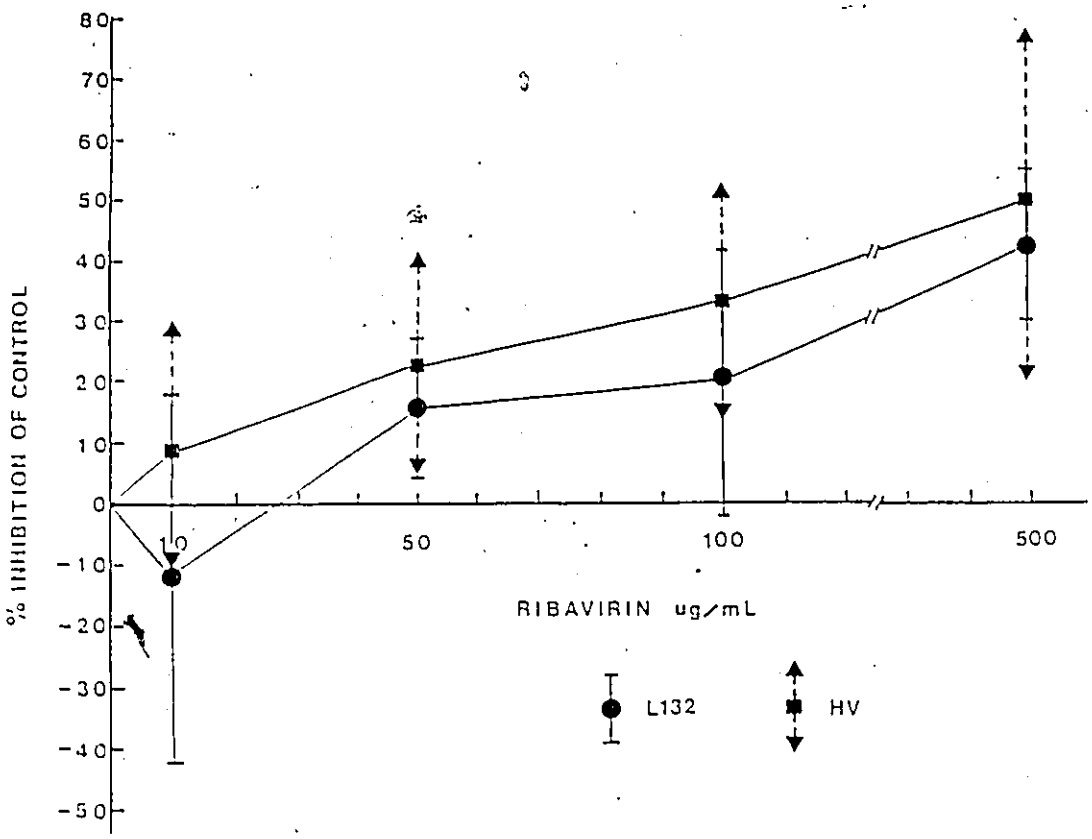


Figure 5. Effect of different concentrations of ribavirin on the % inhibition of total cells/mL on uninfected (L132) and persistently infected (HV) cells at 37°C after 72 h.

Each value represented mean ± S. D. of % inhibition calculated from 3 experiments.


80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
% INHIBITION OF CONTROL
10
50
100
500
RIBAVIRIN ug/mL
L132
HV

2. EFFECT OF RIBAVIRIN ON HUMAN CORONAVIRUS REPLICATION, IN VITRO.

i. Effect of ribavirin on 229E and VH acute infections.

On the basis of the previous results, ribavirin test concentrations from 10-100 ug/mL were chosen. Experiments were designed to determine the inhibitory effect of ribavirin on virus production in L132 cells acutely infected with 229E or VH virus. To help elucidate the effect of ribavirin on virus replication, it was necessary to distinguish whether ribavirin exerted its effect on intracellular events in virus replication or solely on virus release whereby, normal quantities of virus would be detectable within the cells. Therefore, the titers of released virus present in the supernatant fluids (SNFs) as well as cell bound virus—in the trypsinized cell samples were determined. Total virus titers were the combination of both. Because of the demonstrated reduction in cell numbers, it was important to evaluate virus production per cell to learn whether a decrease in virus yield might be due to fewer cells as well as the direct antiviral effect of ribavirin. Total PFU/cell ratios were calculated to evaluate and confirm the antiviral effect of ribavirin.

The results are summarized in Tables 9 and 10. As shown in Table 9, in the 229E acute infection, at MOIs of 1.0-2.5, after a 48 h exposure to ribavirin, there was a significant decrease in the amount of virus production. It was apparent that there was a direct dose-response relationship between the concentrations of ribavirin and the total virus production. This direct dose-dependent response of the total virus production was closely paralleled by the reduction in amounts of cell bound virus. There was a more gradual drop in the amount of released virus present in the SNF, indicating that ribavirin had a direct inhibi-

Table 9. The effect of ribavirin on human coronavirus (229E) acute infection after 48 h. at 33°C*.

RIBAVIRIN ug/mL	RELEASED VIRUS PFU/mLx10^5	CELL BOUND VIRUS PFU/mLx10^5	CELL BOUND VIRUS PFU/cell	TOTAL VIRUS PFU/mLx10^5	TOTAL VIRUS PFU/cell
0	3.0±1.1	45.3±13.3	1.76±0.7	48.3±13.9	1.87±0.7
10	4.1±1.9	16.8±8.8	0.59±0.27	21.0±7.9	0.75±0.2
20	2.7±1.5	11.5±4.3	0.42±0.19	14.2±3.8	0.52±0.15
30	2.0±0.4	7.0±1.9	0.25±0.13	9.0±2.2	0.31±0.1
50	1.01±0.18	3.3±3.1	0.12±0.13	4.3±3.0	0.16±0.13
100	0.8±0.87	0.87±0.4	0.03±0.01	1.7±1.2	0.06±0.03

* Mean of 3 experiments ± S.D.

Table 10. The effect of ribavirin on human coronavirus (VH) acute infection after 48 h. at 33°C*.

RIBAVIRIN ug/mL	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
	PFU/mLx10^7	PFU/mLx10^7	PFU/cell	PFU/mLx10^7	PFU/cell
0	3.1±2.7	6.6±4.7	44.8±35.4	9.7±5.1	59.8±35.6
10	3.8±3.9	5.7±5.5	39.2±38.1	9.5±5.5	61.0±35.2
20	2.2±2.1	2.9±0.7	18.0±4.6	5.1±2.6	29.2±6.1
30	0.76±0.4	2.1±1.2	10.7±5.3	2.8±1.6	14.9±7.2
50	0.27±0.17	0.76±0.7	3.5±3.0	1.02±0.8	4.8±3.5
100	0.21±0.15	0.23±0.15	0.89±0.88	0.44±0.27	2.2±0.8

* Mean of 3 experiments ± S.D.

tory effect on 229E virus replication. At 10 ug/mL ribavirin, there was a 50% reduction in total PFU/mL accompanied by a dramatic decrease in total PFU/cell. As shown in Fig. 6A, 90% inhibition of total PFU/mL was achieved by 50 ug/mL ribavirin. Further analysis of these results showed that the maximum inhibitory effects on the three parameters, released, cell bound, and total virus, occurred between 50-100 ug/mL ribavirin (see also Table 9A, Appendix).

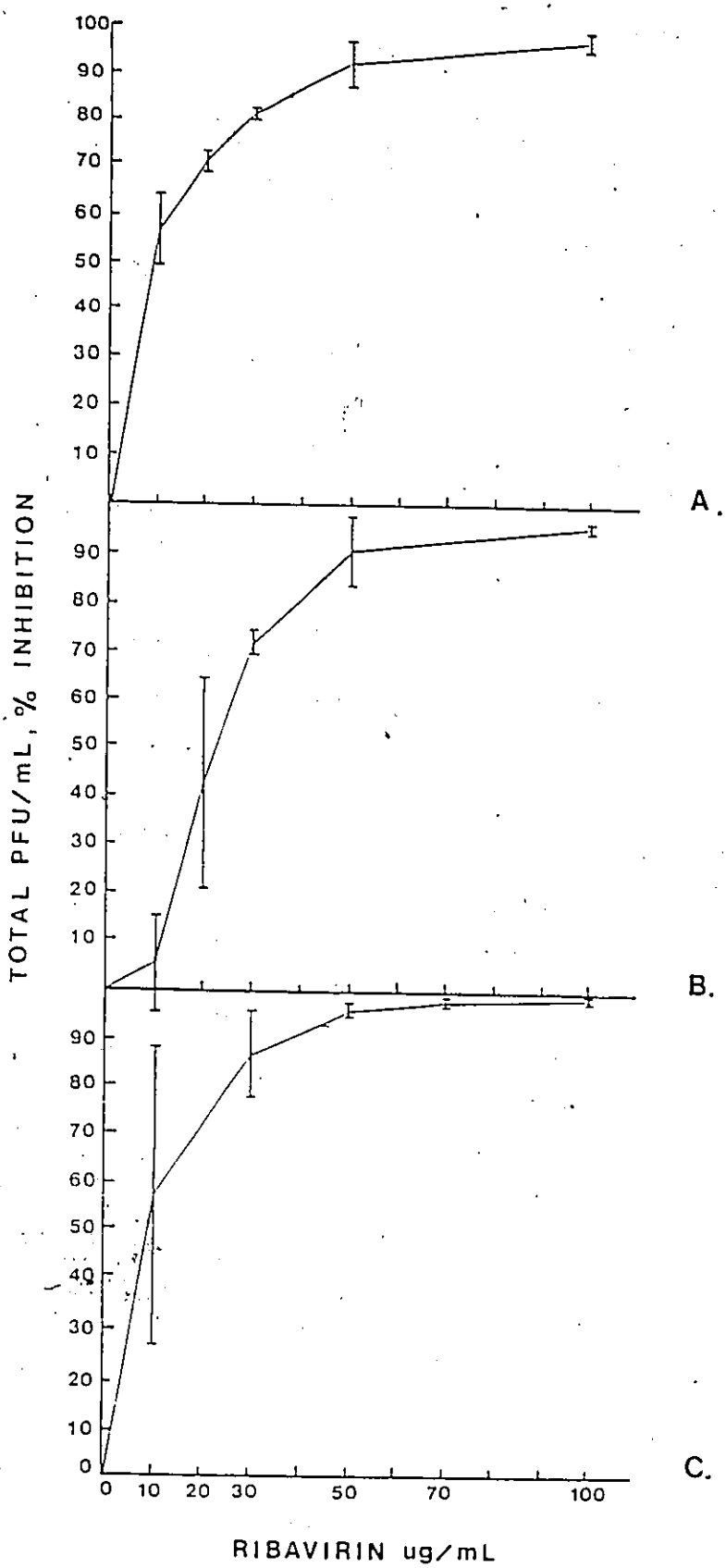
The effect of ribavirin on VH virus production during the acute infection of L132 cells (Table 10) was similar to that found with the 229E system except that 10 ug/mL ribavirin had essentially no effect on VH virus production either on the released or on the cell bound fractions. However from 20-100 ug/mL, as shown in Fig. 6B, there was a marked reduction in total virus which followed a constant and direct dose-response pattern. Further analysis showed that with 50 ug/mL, there was a 90% reduction in total virus production (see also Table 10A, Appendix). Released virus was reduced by approximately 75% at the 20 ug/mL level and maximum reduction occurred around 50 ug/mL. Again, the reduction of cell bound virus paralleled that seen for the total virus indicating that ribavirin exerted its inhibitory effect on intracellular events involved in virus replication and not through blocking the release of progeny virus. Here, the maximum effect was approached at the 100 ug/mL level.

Analysis of variance and covariance with repeated measures was used to evaluate the effect of the different concentrations of ribavirin on the various parameters studied on 229E and VH virus production during acute infection. Statistical tests showed that the differences in the effect of various doses of ribavirin on the production of released, cell

FIGURE 6

Figure 6. Inhibitory effect of ribavirin on total PFU/mL on (A) HCV/229E and (B) HCV/VH acute infection at 33°C, and (C) HV persistent infection at 37°C after 48 h.

Each value represents the mean ± S.D. calculated from three experiments.


TOTAL PFU/mL, % INHIBITION
100
90
80
70
60
50
40
30
20
10
A.
90
80
70
60
50
40
30
20
10
B.
90
80
70
60
50
40
30
20
10
0
C.
0 10 20 30 50 70 100
RIBAVIRIN ug/mL

bound, and total PFU/mL were highly significant for 229E acute infections, with p values of 0.03, 0.00, and 0.00 respectively. In the VH acute infection experiments, the effects of the various concentrations of ribavirin on the total PFU/mL were also significantly different ($p=0.015$). In both 229E and VH studies, the effect of the various concentrations of ribavirin on total PFU/cell ratios differed significantly ($p = 0.0002$ and $p = 0.012$ respectively).

ii. Antiviral activity of ribavirin on HCV persistent infection.

Ribavirin had a similar effect on the amount of VH virus shed from the persistently infected HV cells (Table 11). After 48 h exposure to the drug, even with 10 and 30 ug/mL doses, there was a dramatic decrease in virus yields both in SNFs (cell released) and in cell fractions (cell bound). This inhibitory effect showed a direct dose-response relationship between the concentrations of ribavirin and the reduction of virus shedding. It can be seen that, at 10 ug/mL ribavirin, regardless of the wide deviations due to the variation in amounts of cell bound virus, there was greater than 50% reduction in total PFU/mL and total PFU/cell ratios (See also Table 11A, Appendix). Ribavirin at 50 ug/mL reduced total virus production by greater than 90% (Fig. 6C). The results of statistical tests confirmed that the inhibitory effect of various concentrations of ribavirin on the total PFU/cell ratios was significantly dose dependent ($p = 0.0001$).

In order to compare the effect of ribavirin on 229E and VH virus acute infection in L132 cells and in HV persistently infected cells, the production of virus, measured as PFU/cell over 48 h was plotted as shown in Figure 7. It can be clearly seen that the total PFU/cell ratios, regardless of the different amounts of virus involved, followed the same

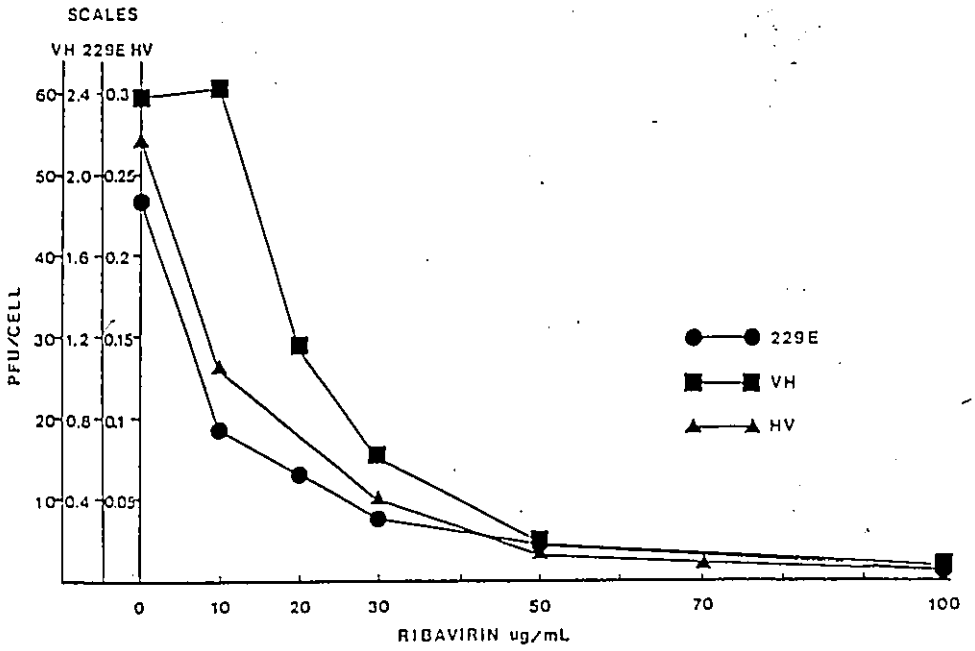
Table 11. The effect of ribavirin on the human coronavirus persistent infection (HV) after 48 h. at 37°C.*

RIBAVIRIN	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
ug/mL	PFU/mLx10^4	PFU/mLx10^4	PFU/cell	PFU/mLx10^4	PFU/cell
0	12.91±7.7	67.45±75.4	0.19±0.1	80.36±78.9	0.27±0.06
10	1.96±0.9	27.13±24.9	0.11±0.09	29.09±25.8	0.13±0.09
30	0.59±0.4	14.92±19.5	0.045±0.04	15.51±19.8	0.05±0.04
50	0.42±0.4	2.35±1.9	0.013±0.008	2.78±2.2	0.02±0.006
70	0.34±0.4	0.97±1.0	0.006±.0004	1.31±1.36	0.008±0.003
100	0.32±0.4	0.83±1.1	0.004±.004	1.15±1.6	0.006±0.004

* Mean of 3 experiments ± S.D.

FIGURE 7

Figure 7. Comparison of the effect of ribavirin on the reduction of total PFU/cell ratios in 229E and VH acute and HV persistent infections.


SCALES
VH 229E HV
60
50
40
30
20
10
2.4
2.0
1.6
1.2
0.8
0.4
0.3
0.25
0.2
0.15
0.1
0.05
PFU/CELL
0
10
20
30
50
70
100
RIBAVIRIN ug/mL
229E
VH
HV

dose-dependent pattern of inhibition due to the antiviral action of ribavirin. At 50 ug/mL of ribavirin optimal antiviral activity was reached.

Quadratic regression was used to analyse the inhibition of total PFU/cell by ribavirin in HCV acute and persistent infections. The log scale regression curves and formulae are shown in Fig. 8. On the basis of these results, the antiviral effect of ribavirin was confirmed and, of the two viruses tested, VH virus in the acute infection system seemed to be more resistant to its action.

3. EFFECT OF RIBAVIRIN ON 229E AND VH CORONAVIRUS PLAQUE FORMATION.

One of the standard methods routinely used for the screening of antiviral drugs is the plaque reduction assay. We therefore measured the effect of different concentrations of ribavirin on HCV plaque formation with various quantities of 229E and VH virus inoculum. The results are summarized in Tables 12 and 13 (also see Tables 12A, and 13A, Appendix).

It is clear that there was a relationship between the dose of ribavirin and the inoculum of virus. Furthermore, if the input 229E or VH virus titres were greater than 8.1×10^4 PFU/mL or 2.4×10^5 PFU/mL respectively, it can be seen that drug concentrations higher than 70 ug/mL would be required. At lower virus input, however, ribavirin exerted a marked anti-plaquing effect, and 50 ug/mL ribavirin in the agar overlay was effective in achieving 90% or greater reduction in plaque formation by both viruses. With 1 ug/mL of the drug and a virus input of 240 PFU/mL, there was 10% inhibition of VH virus plaque formation, while with 8.1 PFU/mL of 229E there was no inhibition. Thus, plaque formation as opposed to replication by VH virus was more sensi-

FIGURE 8

Figure 8. Effect of ribavirin on human coronavirus production. The curves shown opposite were derived from the quadratic regression analysis of the Total PFU/cell ratios. The formulae for each were as follows:

A. 229E acute infection $[2.59-2.54(\log \text{conc.})+2.36(\log \text{conc.})^2]$

Multiple $R^2 = 0.83911$

B. VH acute infection $[121.49-89.35(\log \text{conc.})-30.08(\log \text{conc.})^2]$

Multiple $R^2 = 0.56838$

C. HV persistent infection $[0.33-0.41(\log \text{conc})+0.027(\log \text{conc.})^2]$

Multiple $R^2 = 0.85598$

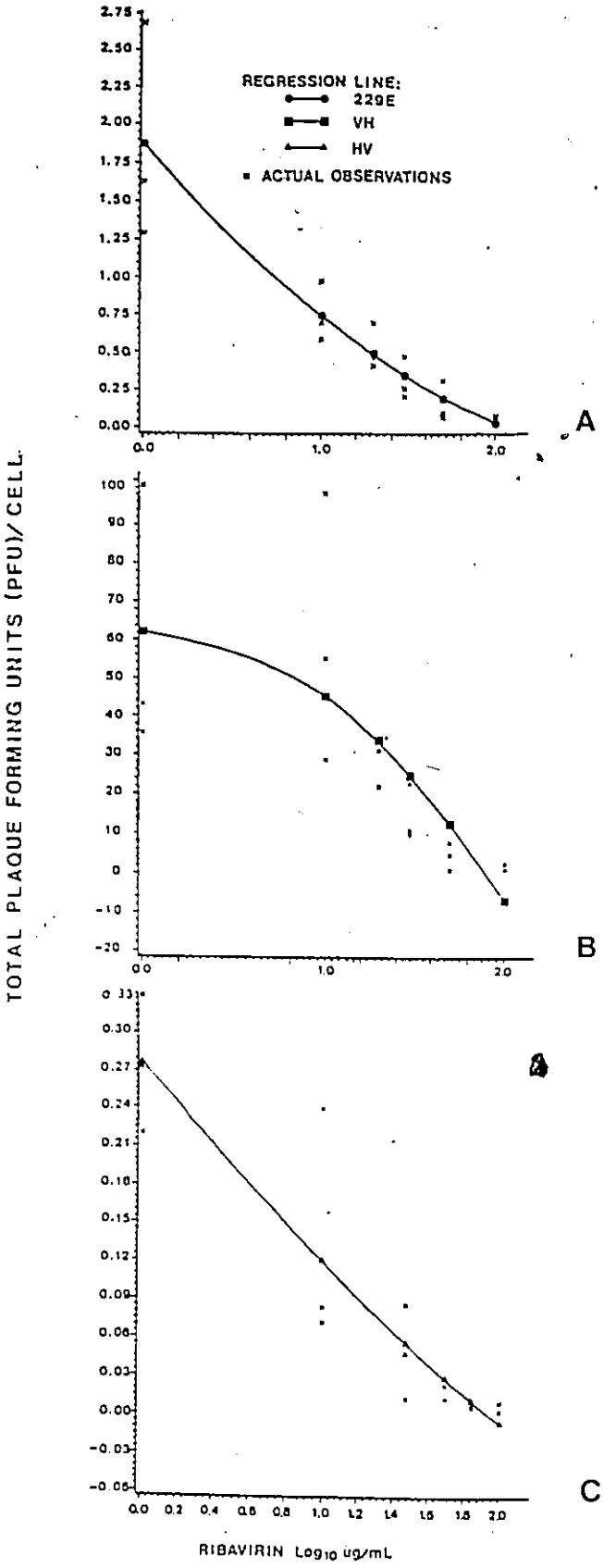

REGRESSION LINE:
229E
VH
HV
ACTUAL OBSERVATIONS
A
B
C
TOTAL PLAQUE FORMING UNITS (PFU)/ CELL
RIBAVIRIN Log10 ug/mL

Table 12. The inhibition of HCV/229E plaque formation by ribavirin.

RIBAVIRIN ug/mL / 229E INOCULUM PFU/mL	% INHIBITION OF PLAQUES*					
	1	10	20	30	50	70
$8.1x10^4$	0	0	0	0	0	99.9**
8100	0	0	0	0	98.3**	100
810	0	0	0	0	92.8**	95.0**
81	***	14.5	24.6	63.8	97.1**	100
8.1	(*)	(*)	(*)	(*)	100	100

* Average of two experiments. Underlined data indicate results obtained from one experiment only. Average numbers of plaques are shown in Table 12A (Appendix).

** Small and indistinct plaques were observed.

(*) 1-4 plaques occurred at this multiplicity and were too few to calculate % inhibition.

*** There was an increase in plaque numbers.

Table 13. The inhibition of HCV/VH plaque formation by ribavirin.

RIBAVIRIN ug/mL / VH INOCULUM PFU/mL	% INHIBITION OF PLAQUES*					
	1	10	20	30	50	70
2.4×10^5	0	0	0	0	0	0
2.4×10^4	0	0	0	0	0	99.7**
2400	0	0	0	0	96.8**	99.5**
240	11.2	6.6	18.3	20.7	0	100
24	15.0	15.0	15.0	100	100	100
2.4	(*)	(*)	(*)	100	100	100

* Average of two experiments. Underlined data indicate results obtained from one experiment only. Average numbers of plaques are shown in Table 13A (Appendix).

** Small and indistinct plaques were observed.

(*) 1-2 plaques occurred at these multiplicities and were too few to calculate % inhibition.

tive to ribavirin than plaque formation and replication by 229E.

In these experiments we also found that ribavirin not only reduced the number of plaque-forming units but also the size of the plaques. Very faint, indistinct micro-plaques were observed in the presence of 50 ug/mL or higher concentrations of ribavirin when input multiplicities of 229E and VH viruses were equal to or greater than 81 and 240 PFU/mL, respectively. These faint, tiny plaques or hazy areas were uncountable, but were clearly different from the uninfected control monolayers.

4. EFFECT OF GUANOSINE ON THE ANTIVIRAL ACTIVITY OF RIBAVIRIN.

Guanosine has been reported to be capable of inhibiting the antiviral activity of ribavirin when used at 200 ug/mL (Streeter et al., 1973). We therefore attempted to inhibit the antiviral effect of ribavirin by guanosine to determine if the compound was exerting its inhibitory effects on the nucleotide pathways to RNA. Also, in the tests on the inhibition of viral replication by ribavirin, low concentrations of the drug which may interfere are probably carried over in the test samples. Therefore, it was of value to see if we could neutralize the potential effects of residual drug in samples if necessary.

It was first necessary to test the effect of guanosine on the host cells used in this study. As can be seen in Tables 14 and 15, guanosine (200 ug/mL) caused a significant reduction in the viable cell numbers of both L132 and HV cells over the 72 h test period. This inhibitory effect was more pronounced on the HV cells as compared to L132 cells, and again, cell counts indicated wider fluctuations in the persistently infected system.

The purpose of the next series of experiments was to examine the

Table 14. The effect of guanosine on the replication of L132 cells grown at 37°C.

	HOURS POST TREATMENT		
	24	48	72
Control	29.2*	38.2	47.7
Guanosine 200 ug/mL	20.1 (30.7±7.2)	31.0 (29.3±10.0)	26.1 (45.1±3.7)

* Mean viable cells x 10^5 from 3 experiments.
( ) Mean ± S.D. of % inhibition calculated for 3 experiments.

Table 15. The effect of guanosine on the replication of HV cells grown at 37°C.

	HOURS POST TREATMENT		
	24	48	72
Control	14.1*	25.3	29.5
Guanosine 200 ug/mL	6.8 (32.8±58.3)	8.8 (57.9±22.3)	10.5 (58.3±11.2)

* Mean viable cells x 10^5 from 3 experiments.
( ) Mean ± S.D. of % inhibition calculated for 3 experiments.

possibility of using guanosine in plaque assays to neutralize residual ribavirin. This was important because, as suggested above, the results of previous replication experiments could have been affected by antiviral activity of ribavirin carried over in the test samples. The concentration of guanosine was chosen on the basis of results reported by Streeter et al. (1973).

As shown in Table 16, ribavirin (50 ug/mL) completely inhibited plaque formation by either 229E or VH viruses, which when there was no drug incorporated in the agar overlay (controls) produced plaques characteristic of their strains. Guanosine alone (200 ug/mL) caused a 20-25 % reduction in plaque numbers in the case of both viruses. In the presence of both ribavirin (50 ug/mL) and guanosine (200 ug/mL) the number of 229E plaques was further reduced but those due to VH virus increased slightly compared to guanosine alone (see also Table 16A, Appendix). Also, the appearance of the plaques of both 229E and VH viruses which developed in the presence of both drugs was very different; although the plaques were well-defined, they were approximately 50-75% smaller than the respective control plaques.

These results did not indicate a significant counteraction by guanosine on the inhibitory activity of ribavirin. Further experiments were carried out to determine if similar results would be obtained in the production of virus by the acute and persistently infected systems. As described in the Materials and Methods section, L132 cells were infected with 229E or VH viruses (MOI 0.2-0.7 and 15-30, respectively) and incubated at 33°C in medium containing either no additives, ribavirin (50 ug/mL), guanosine (200 ug/mL) or both ribavirin and guanosine (50 ug/mL, 200 ug/mL). The results are shown in Tables 17 and 18.

Table 16. The effect of guanosine on the antiviral activity of ribavirin on 229E and VH virus plaque formation.

RIBAVIRIN 50 ug/mL	GUANOSINE 200 ug/mL	NUMBER OF PLAQUES* 229E	VH
-	-	16.2±6.2	11.3±2.5
+	-	0	0
-	+	12.5±3.4	7.3±3.8
+	+	9.5±1.9**	8.7±3.1**

* Mean ± S.D. of 3 experiments. Inocula for 229E and VH ranged between 40-70 PFU/mL.
** Small plaques observed.

Table 17. The effect of guanosine on the antiviral activity of ribavirin on HCV/229E acute infection of L132 cells after 48 h at 33°C*.

RIBAVIRIN	GUANOSINE	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
50 ug/mL	200 ug/mL	PFU/mLX10^5	PFU/mLX10^5	PFU/CELL	PFU/mLX10^5	PFU/CELL
-	-	3.57±2.2	10.27±2.4	0.43±0.2	13.83±3.6	0.54±0.24
+	-	0.47±0.4	1.03±0.6	0.05±0.03	1.50±0.8	0.06±0.04
-	+	6.03±2.9	20.73±3.7	1.08±0.9	26.70±6.6	1.42±1.23
+	+	5.27±1.9	16.00±8.2	0.76±0.6	21.27±8.7	0.96±0.66

* Mean ± S.D. of 3 experiments.

Table 18. The effect of guanosine on the antiviral activity of ribavirin on HCV/VH acute infection of L132 cells after 48 h at 33°C*.

RIBAVIRIN	GUANOSINE	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
50 ug/mL	200 ug/mL	PFU/mLX10^7	PFU/mLX10^7	PFU/CELL	PFU/mLX10^7	PFU/CELL
-	-	2.87±0.9	3.27±1.1	28.83±19.1	6.14±2.0	54.00±35.6
+	-	0.11±0.02	0.22±0.13	1.07±0.7	0.33±0.15	1.50±1.1
-	+	3.57±1.8	4.40±2.2	31.27±22.3	7.97±3.9	55.50±39.5
+	+	3.70±1.06	4.97±1.8	28.53±17.8	8.67±2.75	48.67±28.4

* Mean ± S.D. of 3 experiments.

It can be seen that in 229E and VH acute infection, the antiviral effects of ribavirin were blocked by addition of guanosine. In the presence of guanosine alone, or both ribavirin and guanosine, there was no reduction of the virus yields in both SNF and cell samples. In fact, the virus production associated with all the parameters examined actually increased considerably compared to the controls. Again, ribavirin (50 ug/mL) exerted the expected reduction in virus produced by the acute infection system (approximately 90% for both 229E and VH).

We next tested the ability of guanosine to interfere with the antiviral activity of ribavirin on the HV persistently infected cells using the same experimental protocol. The results are shown in Table 19. It can be clearly seen that guanosine did not have the same ribavirin-blocking activity demonstrated in the acute infections. In this persistent infection system, guanosine alone markedly inhibited total virus production. The degree of antiviral activity of guanosine (200 ug/mL) was as great as that of 50 ug/mL of ribavirin. In the presence of both ribavirin and guanosine, there were no changes in the level of inhibition under the conditions of these experiments.

A comparison of all the results of the experiments to determine the ability of guanosine to prevent the inhibitory effect of ribavirin during virus production by either the acute or persistent systems is presented in Fig. 9. The differences in the responses of the acute versus the persistent infections is clearly evident. Specifically, guanosine is potentially of value in counteracting the inhibition of ribavirin, on both the 229E and VH acute infections, although its presence seemed to contribute to wide variations in virus production. In the case of the HV persistent infection, use of guanosine had a

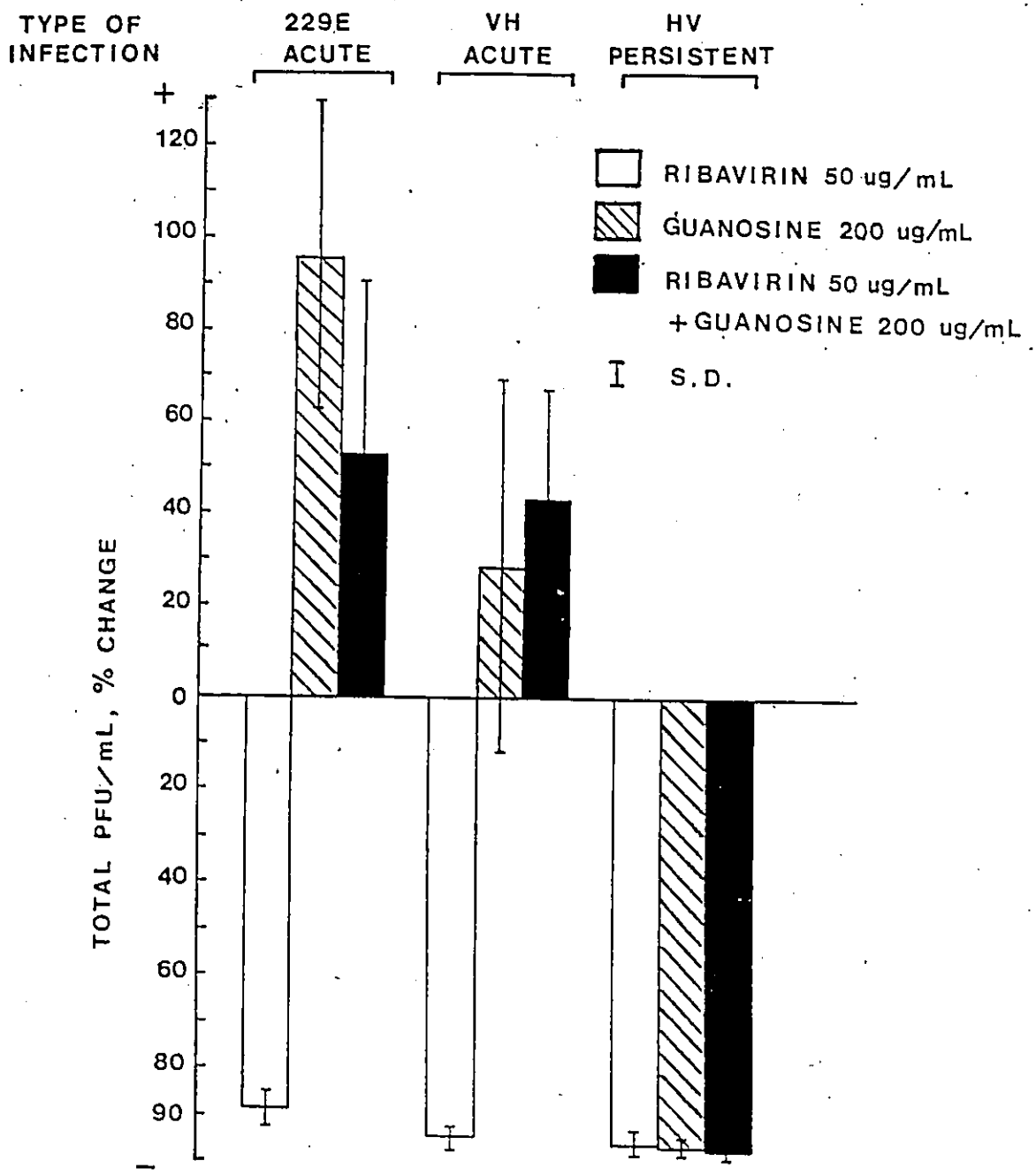
Table 19. The effect of guanosine on the antiviral activity of ribavirin on the HV persistent infection after 48 h at 37°C.*

RIBAVIRIN	GUANOSINE	TOTAL VIRUS	
50 ug/mL	200 ug/mL	PFU/mLx10^4	PFU/cell
-	-	44.3	0.20
+	-	1.2 (97.3)	0.006 (97.0)
-	+	0.87 (98.0)	0.01 (95.0)
+	+	0.84 (98.1)	0.0085 (95.7)

* Average of two experiments.
( ) % inhibition.

FIGURE 9

Figure 9. Comparison of the effect of guanosine on the antiviral activity of ribavirin on human coronavirus production during HCV acute and persistent infections.


TYPE OF INFECTION
229E ACUTE
VH ACUTE
HV PERSISTENT
RIBAVIRIN 50 ug/mL
GUANOSINE 200 ug/mL
RIBAVIRIN 50 ug/mL +GUANOSINE 200 ug/mL
S.D.
TOTAL PFU/mL, % CHANGE
+
120
100
80
60
40
20
0
20
40
60
80
90
-

pronounced deleterious effect.

5. ATTEMPTS TO CURE PERSISTENTLY INFECTED HV CELLS USING RIBAVIRIN.

An experiment aimed at curing the HV cells of their persistent infection by continuous passage of the cells in the presence of ribavirin was carried out according to the procedure described in the Materials and Methods section. 10 and 50 ug/mL of ribavirin were chosen for testing because at these concentrations ribavirin exerted a significant antiviral effect but did not markedly affect host cell replication. The cells did not form good monolayers in the presence of 50 ug/mL ribavirin because of drug cytostatic effect however, it was possible to successfully maintain the cultures over 7 passages. The results of the plaque assays of both cell bound and released viruses at each time of cell passage for the duration of this curing experiment are presented in Fig. 10 A & B (also see Table 20, Appendix). In the absence of ribavirin the control cultures continued to produce the usual amounts of virus and although there was a slight drop in both cell bound and released virus produced by cells treated with 10 ug/mL ribavirin, this was apparently transient, and after 3-4 cell passages had returned to control levels.

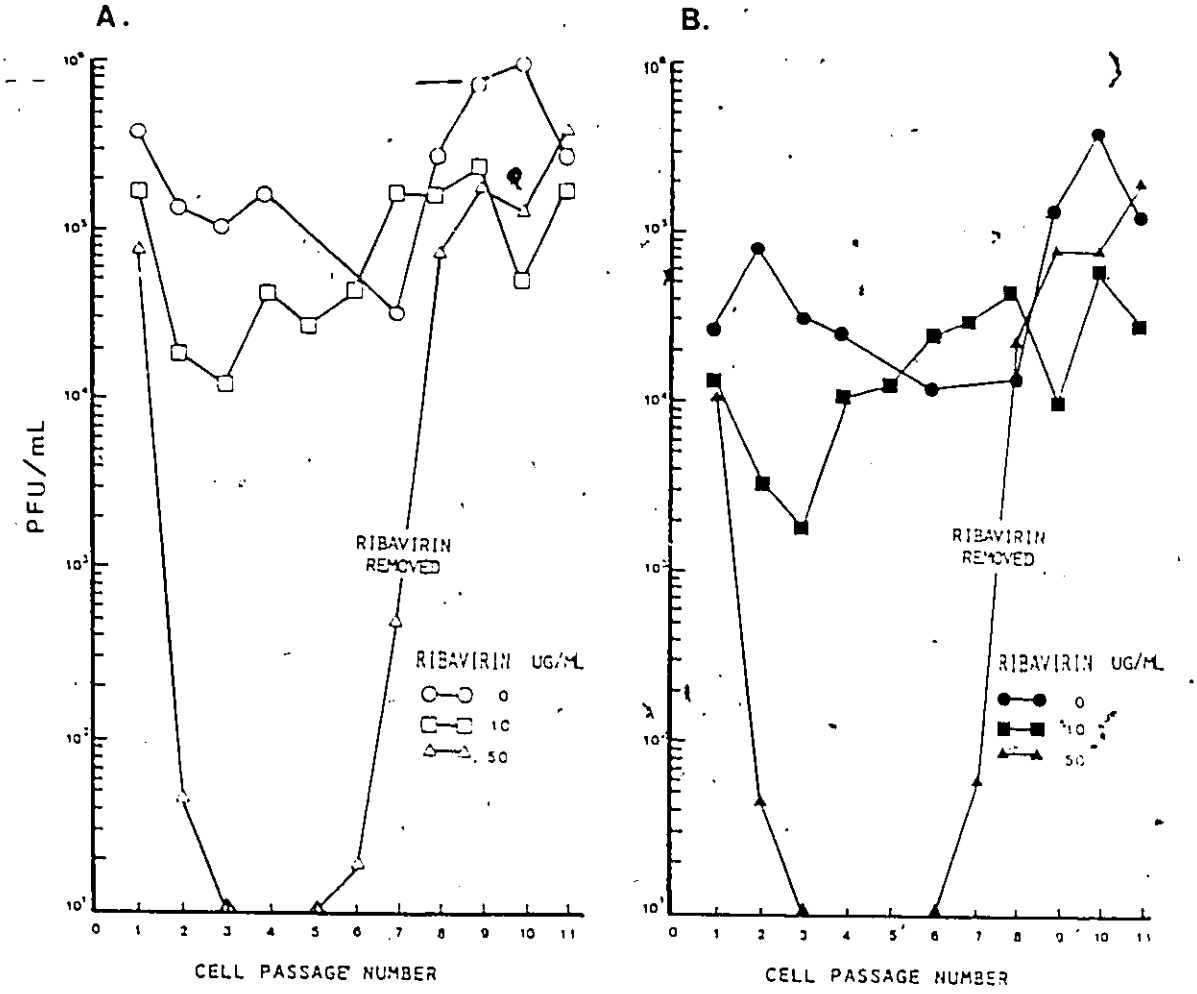
On the other hand, it can be clearly seen that after 3 cell passages in the presence of 50 ug/mL of ribavirin, no virus was detected either in the SNF or in cell samples. But, because the plaque assay of the samples required 5 days, there was a minimum lag of 3 cell passages before deciding whether the elimination of virus from the cells was successful or not. Thus, the decision to remove the drug from the culture was made half way through the 7th cell passage when the results of the first four cell passages indicating elimination of virus were

FIGURE 10

Figure 10. Effect of ribavirin on virus production (PFU/mL) during 11 passages of persistently infected HV cells.

(A) cell bound virus.

(B) released virus.


A.
B.
PFU/mL
RIBAVIRIN REMOVED
RIBAVIRIN UG/ML
0
10
50
CELL PASSAGE NUMBER

obtained. As can be seen in Figure 10, virus production in both SNFs and cells resumed normal levels within 24 hours. However, that the decision was premature, was evident from the results obtained from the next series of plaque assays. The cultures had remained virus-free until the 5th cell passage when, still in the presence of 50 ug/mL ribavirin, low levels of cell bound virus appeared. Furthermore, at cell passage 6, virus was detectable in the SNF and increasing amounts were being shed after 7 cell passages.

These results indicated a loss of sensitivity to the antiviral activity of ribavirin. These 'ribavirin-conditioned' cultures were continuously maintained and monitored for 23 passages after this experiment. They continued to shed similar amounts of infectious virus as the control HV persistently infected cells and the virus derived therefrom (VH-Rc) appears to share the VH characteristics. Preliminary results indicated that the virus shed at passage 7 was more resistant to the effects of 50 ug/mL ribavirin than the VH virus stock (VH-Rc7 produced 407 PFU/mL compared to 0 PFU/mL of VH virus). However, the resistance characteristics of the virus being shed post ribavirin treatment (VH-Rc virus) have not been evaluated.

IV. DISCUSSION

Ribavirin, a synthetic inhibitor of RNA and DNA virus replication, has displayed considerable chemotherapeutic activity against experimentally induced viral infections and currently is undergoing extensive clinical evaluations in humans. Some of its most promising features are a broad spectrum of activity (the broadest of the currently studied antiviral agents), a relative lack of toxicity for normal or virus-infected host cells, and the failure of virus strains exposed to ribavirin to develop drug resistance. There have been however, no published reports on the effect of ribavirin on coronavirus infections and this study was undertaken to fill that gap.

Ribavirin has also been reported to inhibit cellular DNA synthesis in KB cells (Drach and Shipman, 1977), and to affect proliferation and macromolecular synthesis in mouse lymphoma cells (Muller et al., 1977). These in vitro results suggested that ribavirin is a chemotherapeutic agent with both a cytostatic and an antiviral activity.

The report by De Clercq et al. (1975) suggested that the antiviral effect of ribavirin might be a result of an inhibition of nucleic acid synthesis in infected cells. It has been reported that ribavirin is a competitive inhibitor of DNA and RNA synthesis and at high concentrations has a static and somewhat enlarging effect on cells in vitro, however, it does not appear to be incorporated into cellular materials (Sidwell et al., 1977). The data presented here indicate that ribavirin inhibited the multiplication of both L132 uninfected and HV persistently infected cells with a 50% inhibitory concentration which was equal to or greater than 100 ug/mL over a 72 h period. Neither of the cell types

showed any noticeable cytotoxic changes and the viability of both was not affected significantly by the ribavirin treatment. Thus, our results supported those of others and we concluded that ribavirin has a cytostatic but not cytotoxic effect and that concentrations in the 50 ug/mL range were appropriate for further testing.

Our studies have demonstrated the antiviral action of ribavirin on the replication of both the prototype 229E and the persistent virus, VH. During HCV 229E and VH acute infections, the yield of virus released from cells as well as the cell associated virus was markedly reduced by ribavirin at concentrations ranging from 10 to 50 ug/mL. It appeared that VH virus was more resistant to the action of ribavirin than 229E, however, this conclusion is preliminary and requires further study. There was also a marked reduction of virus shedding by the persistently infected HV cells with the 50% inhibitory concentration of ribavirin being in the 10-30 ug/mL range. In all systems the antiviral activity was also reflected by a dramatic decrease in total PFU/cell ratios which was directly associated with increasing drug concentrations. These results were interpreted to mean that in spite of the cytostatic effect, there was a direct inhibition of virus production in the cells due to the antiviral activity of ribavirin. Statistical analysis of these results confirmed that there was a direct dose-response relationship between the concentration of ribavirin and virus yields.

Further studies were undertaken to evaluate the effect of ribavirin using the plaque reduction assay. This is one of the standard procedures used for testing antiviral activity and these experiments were designed to provide information on the relationship between the inhibitory dose of ribavirin and the input virus multiplicity. In addition,

the determination of the minimal inhibitory concentration (MIC) of ribavirin for each of the viruses would provide another parameter for comparing them. Our results demonstrated that ribavirin could inhibit plaque formation by both viruses, however, closer examination of the data revealed some unexpected differences between the two.

In the first place, the 50% inhibitory dose for the higher input multiplicities ($>10^4$) was greater for 229E than for VH virus implying that, contrary to the results on virus production, 229E was the more resistant virus. There also was a fairly consistent dose-response relationship between the decreasing drug concentrations and decreasing input multiplicity of 229E. This was not the case with VH virus where there was a stepwise type of relationship with a sharp difference in virus sensitivity which occurred with input multiplicities between 300 - 3000 in the 30-50 ug/mL ribavirin dose range. Furthermore, there was no progressive decrease at the lower input multiplicities. On the basis of these results, it might be concluded that there is a sub-population of VH virions which are highly sensitive to the antiviral activity of ribavirin. Comparison of the ribavirin MICs which might be calculated from the results obtained at low input multiplicities would suggest that again, in contrast to the earlier conclusion, VH virus was the more sensitive strain. It must be pointed out, however, that these results are a reflection of the plaque-forming capabilities of the test viruses which are not necessarily directly related to virus replication in the cells.

The ribavirin effect on plaque formation was characterized by the marked reduction in the plaque sizes and was associated with concentrations of ribavirin greater that 50 ug/mL which inhibited by

50% or more. Also, at the higher input multiplicities, where control plaque results were too numerous to count, there was a definite difference between the ribavirin treated cell monolayers and untreated controls. This was interpreted as indicating that virus was present but that there was reduced cell destruction required for the production of plaques.

Other workers have reported that ribavirin affected the plaque characteristics of viruses used in their studies. Oxford (1975) found that the plaque size of fowl plaque virus was significantly smaller in the presence of ribavirin at a concentration of 0.1 mM (24.4 ug/mL), the lowest dose tested. Also, Hruska et al. (1980) found that in the presence of ribavirin at 10 ug/mL or higher, small plaque morphology was observed for all respiratory syncytial virus strains tested. As a result of our experiments, we conclude that ribavirin had a direct, dose-dependent inhibitory effect on the plaque formation by 229E virus and that at the higher multiplicities, plaque formation by 229E was more resistant to ribavirin than VH virus. The results obtained in the VH system suggest that there are some basic differences between the two virus strains which warrant further investigation. Finally, it was concluded that reliance on the results of plaque reduction assays only for the evaluation of the antiviral activity of ribavirin could give misleading results.

However, it is not yet clear from the literature whether ribavirin is specifically antiviral in its mode of action or whether it inhibits virus replication as a result of its effect on the host cell. On the basis of our results so far, we cannot rule out the involvement of a ribavirin sensitive host cell contribution to the antiviral effect.

We suggest that there is evidence for the direct antiviral action indicated by the reduction in PFU/cell ratios.

The next stage in our studies involved the used of guanosine to determine whether it could be used for reversing or 'neutralizing' the inhibitory action of ribavirin. Although guanosine had been shown to reverse the action of ribavirin in measles virus-infected Vero cells (Streeter et al., 1973), and in fowl plaque virus infected CEF cells (Oxford, 1975), in our studies guanosine was only completely effective in neutralizing the antiviral activity of ribavirin on the production of HCV 229E and VH viruses during acute infections. In the experiments on the ribavirin inhibition of 229E and VH plaque formation, guanosine was only partially successful in abrogating the response. When tested on the HV persistent infection guanosine was totally unsuccessful. This failure of guanosine to block the action of ribavirin in the HV cells is likely due to the fact that by itself, guanosine at the concentration of 200 ug/mL, inhibited HV cell replication and virus production. Malinoski and Stollar (1981) reported that in Aedes albopictus mosquito cells, equimolar amounts of guanosine ($6x10^{-4}M$) did not reverse the inhibition of Sindbis virus replication by ribavirin and they noted that in these cells, guanosine by itself, was inhibitory. We also found that guanosine, at the concentration tested (200 ug/mL), inhibited L132 cell replication. However, in contrast, 200 ug/mL guanosine did not inhibit 229E and VH virus production by L132 cells. In fact, it tended to enhance virus production. When used together with ribavirin, guanosine successfully counteracted the antiviral activity of ribavirin.

The mechanisms responsible for the different effects of guanosine both acting alone and on the antiviral activity of ribavirin on the 229E

and VH infections need further study. Since the antiviral effect of ribavirin could be readily reversed by guanosine in the HCV acute infections its use in the study of early replicative events in HCV infected cells may be of value.

The effectiveness of ribavirin against an established human coronavirus persistent infection was tested to evaluate the potential of the drug to cure the cells by eliminating the virus. After the first three cell passages in the presence of ribavirin (50 ug/mL), the results were very encouraging because virus production appeared to be eliminated. However, with further passage of the cells, still in the presence of ribavirin, low levels of virus began to appear; first in the cell fractions followed one passage later in the SNF. And with the removal of ribavirin, virus production immediately resumed normal levels, indicating that a cure had not been obtained. It is possible that longer treatment and/or higher doses could effect a complete cure in vitro or combination of ribavirin with anti-HCV serum, or with other antiviral drugs could enhance the antiviral activity in curing HV cells of their persistent infection. However, of greater interest, is the nature of the virus shed under the influence of ribavirin because of the possible development of a virus with ribavirin resistance properties. Our preliminary results showed that the virus shed in the 7th cell passage was more resistant to 50 ug/mL ribavirin. These results are of great interest and further experiments are needed to determine whether they are stable ribavirin-resistant mutants or not.

The present studies demonstrate that ribavirin had marked antiviral activity against both HCV acute and persistent infections. The applicability of these in vitro results to the treatment of human

infections due to HCV is not straightforward. These results, however, are of value in confirming and expanding positive results of primary screening tests and thus provide an important link between screening in vitro and animal experiment or clinical trials.

V. GENERAL CONCLUSIONS

The main purpose of this study was to determine the effect of ribavirin on uninfected (L132) and persistently infected (HV) cell replication and to evaluate the antiviral activity of ribavirin on human coronavirus production both in HCV acute and persistent infections and on plaque formation. It was hoped that this might help us to understand the mode of action of ribavirin, whether it is specifically antiviral or whether it inhibits coronaviral replication as a result of its effect on the host cell.

Our data demonstrated that ribavirin had a marked effect on both L132 and HV cell replication. Drug concentrations of 100-500 ug/mL induced a reduction in the numbers of total cells of both cell lines although the cell monolayers did not show any noticeable cytotoxic effects. Analysis of the percentage of viable cells of both cell types showed that the cell viability did not change significantly after ribavirin treatment. Thus it was concluded that ribavirin was a cytostatic but not a cytotoxic agent.

The present results also showed that ribavirin had a pronounced antiviral effect on the production of human coronavirus in an acute infection, in vitro. A constant dose-response relationship between the concentrations of ribavirin and the yields of virus was observed. Ribavirin also exhibited significant in vitro antiviral activity against the HV persistently infected cells inducing a marked reduction in virus shedding. The VH virus derived from the persistently infected cells, appeared to be more resistant to ribavirin than 229E, but the results varied depending on the assay system used.

A reduction of plaque formation by both 229E and VH viruses was

observed at ribavirin concentrations ranging from 30-50 ug/mL. This anti-plaquing effect was found to be related to the size of the virus inoculum. At concentrations greater than 50 ug/mL, when high MOIs were used, the size of the plaques which developed were markedly decreased. In these circumstances, very hazy, faint, uncountable areas were also observed. Further studies are required to determine whether the viruses in these plaques have acquired an increased resistance to ribavirin.

The antiviral effect of ribavirin, including inhibition of virus production and anti-plaque formation, can be counteracted by the addition of 200 ug/mL guanosine only in HCV acute infections. This might confirm the early hypothesis that ribavirin is a potent competitive inhibitor of inosine 5'-phosphate dehydrogenase, and that the antiviral activity of ribavirin may be mediated by inhibition of GMP biosynthesis at the stage of conversion of IMP to xanthosine 5'-phosphate (Streeter, et al., 1973). In HV persistently infected cells, however, 200 ug/mL guanosine inhibited cell replication as well as virus production. This might support observations that continuous cell metabolism is a requirement for virus production in this persistently infected system. On the other hand, although guanosine could strongly inhibit the replication of L132 cells, this apparently did not affect the blocking of antiviral activity by ribavirin. Further investigations are needed to determine whether these findings indicate differences in the mechanisms of the antiviral activity of ribavirin.

In the curing experiments, ribavirin initially showed an encouraging antiviral activity. In HV persistently infected cells in the presence of 50 ug/mL ribavirin, virus production (both released and cell bound viruses) appeared to be eliminated by the third cell passage and remained virus-free for 2 cell passages. However, there was a resumption

of virus production at cell passage 5 indicating either a loss of sensitivity to ribavirin or the selection of more resistant virions already present in the population.

Attempts to cure the persistently infected cells by treating with ribavirin were complicated by the cytostatic effect of the drug. The fact that virus production resumed after removal of the drug indicated that there was no permanant damage to the cell or to the cell's virus-synthesizing machinery. Our preliminary results suggest that ribavirin is a virustatic agent with an accompanying cytostatic effect. The possibility of the emergence of a ribavirin-resistant mutant during the curing experiment requires further study especially since our preliminary results have shown that the virus shed while still in the presence of ribavirin was more resistant than VH stock virus.

In conclusion, therefore, ribavirin does induce a marked reduction in virus production in both HCV acute infections as well as in an established HCV persistent infection in vitro. Persistent infections with viruses are implicated in several human diseases. Because of the severe consequences of these diseases, further research in the efficacy of ribavirin treatment of persistent viral infections seems warranted.

VI. REFERENCES

Allen, L.B. (1980): Review of in vivo efficacy of ribavirin. In: Ribavirin: a Broad Spectrum Antiviral Agent, edited by R.A. Smith, and W. Kirkpatrick, pp. 43-58. Academic Press, New York.

Allen, L.B. and Fingal, C.M. (1977): Failure of type 1 herpesvirus to develop resistance to ribavirin. Antimicrob. Agents Chemother. 12: 120-121.

Allen, L.B., Wolf, S.M., Hintz, C.J., Huffman, J.H. and Sidwell, R.W. (1977): Effect of ribavirin on type 2 herpesvirus hominis (HVH/2) in vitro and in vivo. Ann. N. Y. Acad. Sci. 284: 247-253.

Almeida, J.D. and Tyrrell, D.A.J. (1967): The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. J. Gen. Virol. 1: 175-178.

Banks, G. and Fernandez, H. (1984): Clinical use of ribavirin in measles: a summarized review. In: Clinical Applications of Ribavirin, edited by R.A. Smith, V. Knight, and J.A.D. Smith, pp. 203-209. Academic Press, New York.

Bradburne, A.F. and Tyrrell, D.A.J. (1969): The propagation of coronaviruses in tissue culture. Arch. Ges. Virusforsch. 28: 133-150.

Burks, J.S., DeVald, B.D., Jankovsky, L.C. and Gerdes, J.C. (1960): Two coronaviruses isolated from central nervous system tissue of two multiple sclerosis patients. Science. 209: 933-934.

Canonico, P.G., Kende, M. and Huggins, J.W. (1984): The toxicology and pharmacology of ribavirin in experimental animals. In: Clinical Applications of Ribavirin, edited by R.A. Smith, V. Knight, and J.A.D. Smith, pp. 65-77. Academic Press, New York.

Chaloner-Larsson, G. and Johnson-Lussenburg, C.M. (1981a): Characteristics of a long term in vitro persistent infection with human coronavirus 229E. In: Biochemistry and Biology of Coronaviruses, edited by V. ter Meulen, S. Siddell, and H. Wege, pp. 309-322. Plenum Press, New York.

Chaloner-Larsson, G. and Johnson-Lussenburg, C.M. (1981b): Establishment and maintenance of a persistent infection of L132 cells by human coronavirus strain 229E. Arch. Virol. 69: 117-129.

De Clercq, E., Luczak, M., Reepmeyer, J.C., Kirk, K.L. and Cohen, L.A. (1975): Fluoroimidazoles as antiviral agents and inhibitors of polynucleotide biosynthesis. Life Sci. 17: 187.

De Clercq, E. and Luczak, M. (1976): Intranasal challenge of mice with herpes simplex virus: an experimental model for evaluation of the efficacy of antiviral drugs. J. Inf. Dis. (Suppl.) 133: A226-A236.

De Clercq, E., Luczak, M., Shugar, D., Torrence, P.F., Waters, J.A. and Witkop, B. (1976): Effect of cytosine, arabinoside, iododeoxyuridine, ethyldeoxyuridine, thiocyanalodeoxyuridine, and ribavirin on tail lesion formation in mice infected with vaccinia virus. Proc. Soc. Exp. Med. 151: 487-490.

Descamps, J. and De Clercq, E. (1978): Broad-spectrum antiviral activity of pyrazofurin (pyrazomycin). In: Current Chemotherapy, vol.1, edited by W. Siegenthaler and R. Luthy, pp. 354-357. (Proc. 10th Int. Congr. Chemother., Zurich). Am. Soc. Microbiol. Washington, D.C.

Dowling, J.N., Postic, B. and Guevarra, L.O. (1976): Effect of ribavirin on murine cytomegalovirus infection. Antimicrob. Agents Chemother. 10: 809-813.

Drach, J.C. and Shipman, C. (1977): The selective inhibition of viral DNA synthesis by chemotherapeutic agent:. an indicator of clinical usefulness. Ann. N. Y. Acad. Sci. 284: 396-409.

Drach, J.C., Thomas, M.A., Barnett, J.W., Smith, S.H. and Shipman, C. (1981): Tritiated thymidine incorporation does not measure DNA synthesis in ribavirin-treated human cells. Science. 212: 549-551.

Fernandez, H. (1980): Ribavirin: a summary of clinical trials --- herpes genitalis and measles. In: Ribavirin: a Broad Spectrum Antiviral Agent, edited by R.A. Smith, and W. Kirkpatrick, pp. 215-230. Academic Press, New York.

Fernandez, H. and Diaz-Perches, R. (1977): Clinical experiences using the antiviral 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) in Mexico. Ann. N. Y. Acad. Sci. 284: 284-288.

Gilbert, B.E. and Knight, V. (1986): Biochemistry and clinical applications of ribavirin. Antimicrob. Agents Chemother. 30: 201-205.

Goswami, B.B., Borek, E., Sharma, O.K., Fujitaki, J. and Smith, R.A. (1979): The broad spectrum antiviral agent ribavirin inhibits capping of mRNA. Biochem. Biophys. Res. Commun. 89: 830-836.

Hall, C.B., McBride, J.T., Walsh, E.E., Bell, D.M., Gala, C.L., Hildreth, S., Ten Eyck, L.G. and Hall, W.J. (1983): Aerosolized ribavirin treatment of infants with respiratory syncytial viral infection, a randomized double-blind study. N. Engl. J. Med. 308: 1443-1447.

Hamre, D. and Procknow, J.J. (1966): A new virus isolated from the human respiratory tract. Proc. Soc. Exp. Biol. Med. 121: 190-193.

Hillyard, I.W. (1980): The preclinical toxicology and safety of ribavirin. In: Ribavirin, a Broad Spectrum Antiviral Agent, edited by R.A. Smith, and W. Kirkpatrick, pp. 59-71. Academic Press, New York.

Holmes, K.V. (1985): Replication of coronaviruses. In: Virology, edited by B.N. Fields, pp. 1331-1343. Raven Press, New York.

Hruska, J.F., Bernstein, J.M., Douglas, R,G. and Hall C.B. (1980): Effect of ribavirin on respiratory syncytial virus in vitro. Antimicrob. Agents Chemother. 17: 770-775.

Huffman, J.H., Allen, L.B. and Sidwell, R.W. (1977): Comparison of the development of resistant strains of type 1 herpes simplex virus to in vitro antiviral activity of 5-iodo-2'-deoxyuridine or ribavirin. Ann. N. Y. Acad. Sci. 284: 233-238.

Huffman, J.H., Sidwell, R.W., Khare, G.P., Witkowski, J.T., Allen, L.B. and Robins, R.K. (1973): In vitro effect of 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Agents Chemother. 3: 235-241.

Jolley, W.B., Call, T.W. and Alvord, L.S. (1977): Immunosuppressive activity of ribavirin using the rabbit skin allograft model. Ann. N. Y. Acad. Sci. 284: 230-232.

Jolley, W.B., Hinshaw, D.B. and Pettis, J.L. (1980): Minimal effects of ribavirin on the immune system. In: Ribavirin: a Broad Spectrum Antiviral Agent, edited by R.A. Smith, and W.Kirkpatrick, pp.73-81. Academic Press, New York.

Jolley, W.B. and Suchil, C. (1984):Effects of ribavirin on the immune system (second report). In: Clinical Applications of Ribavirin, edited by R.A. Smith., V. Knight, and J.A.D. Smith, pp.93-96. Academic Press, New York.

Katz, E., Margalith, E. and Winer, B. (1976): Inhibition of vaccinia virus growth by the nucleoside analogue 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide. J. Gen. Virol. 32: 327-330.

Kennedy, D.A. and Johnson-Lussenburg, C.M. (1975/1976): Isolation and morphology of the internal component of human coronavirus, strain 229E. Intervirol. 6: 197-206.

Kennedy, D.A. and Johnson-Lussenburg, C.M. (1979): Inhibition of coronavirus 229E replication by actinomycin-D. J. Virol. 29: 401-404.

Klassen, L.W., Williams, G.W., Reinersten, J.L., Gerber, N.L. and Steinberg, A.D. (1979): Ribavirin treatment in murine autoimmune disease. I. Therapeutic efficacy and effect on the immune response. Arthritis Rheum. 22: 145-154.

Knight, V., McClung, H.W., Wilson, S.Z., Waters, B.K., Quarles, J.M., Cameron, R.W., Greggs, S.E., Zerwas, J.M. and Couch, R.B. (1981): Ribavirin small-particle aerosol treatment of influenza. Lancet. 2: 945-949.

Lucas, A., Coulter, M., Anderson, R., Dales, S. and Flintoff, W. (1978): In vivo and in vitro models of demyelinating diseases: persistence and host-regulated thermosensitivity in cells of neural derivation infected with mouse hepatitis and measles virus. Virol. 88: 325-337.

Lucas, A., Flintoff, W., Anderson, R., Percy, D., Coulter, M. and Dales, S. (1977): In vivo and in vitro models of demyelinating diseases: tropism of the JHM strain of murine hepatitis virus for cells of neural origin. Cell. 12: 553-560.

Macnaughton, M.R. and Davies, H.A. (1981): Human enteric coronaviruses. Arch. Virol. 70: 301-313.

Malluci, L. (1965): Observations on the growth of mouse hepatitis virus (MHV-3) in mouse macrophages. Virol. 25: 30-37.

Maninoski, P. and Stoller, V. (1981): Inhibits of dehydrogenase prevent Sindbis virus replication and reduce GTP levels in Aedes albopictus cells. Virol. 110: 281-291.

McCammon, J.R. and Riesser, V.W. (1979): Effects of ribavirin on BHK-21 cells acutely or persistently infected with mumps virus. Antimicrob. Agents Chemother. 15: 356-360.

McClung, H.W., Knight, V., Gilbert, B.E., Wilson, S.Z., Quarles, J.M. and Divine, G.W. (1983): Ribavirin aerosol treatment of influenza B virus infection. J. Am. Med. Assoc. 249: 2671-2674.

McCormick, J.B., Getchell, J.P., Mitchell, S.W. and Hicks, D.R. (1984): Ribavirin supresses replication of lymphadenopathy-associated virus in cultures of human adult T lymphocytes. Lancet. 2: 1367-1369.

McCormick, J.B., King, I.J., Webb, P.A., Scribner, C.L., Craven, R.B., Johnson, K.M., Elliott, L.H. and Belmont-Williams, R. (1986): Lassa fever effective therapy with ribavirin. N. Engl. J. Med. 314: 20-26.

McIntosh, K. (1979): Coronaviruses. In: Principles and Practice of Infectious Disease, edited by G.L. Mandell, G.R. Douglas, and J.E. Bennett, pp. 1212-1217. John Wiley and Sons, New York.

Miller, J.P., Kigwana, L.J., Streeter, D.G., Robins, R.K., Simon, L.N. and Roboz, J. (1977): The relationship between the metabolism of ribavirin and its proposed mechanism of action. Ann. N. Y. Acad. Sci. 284: 211-229.

Muller, W.E.G. (1979): Mechanism of action and pharmocology: clinical agents. In: Antiviral Agents and Viral Diseases of Man, edited by G.J. Galasso, T.C. Merigan, and R.A. Buchanan, pp. 77-149. Raven Press, New York.

Muller, W.E.G., Maidhof, A., Taschner, H. and Zahn, R.K. (1977): Virazole (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a cytostatic agent. Biochem. Pharmacol. 26: 1071-1075.

Murphy, M.F. (1978): In vitro inhibition of subacute sclerosing panencephalitis virus by the antiviral agent ribavirin. J. Inf. Dis. 138: 249-251.

Newman, H., Vodinelich, L. and Potter, C.W. (1977): The effects of 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (ribavirin) on the transplanted tumours of animals. European J. Cancer. 13: 821-830.

Oberg, B. and Helgstrand, E. (1978): Selective inhibition of viral polymerase by ribavirin triphosphate. In: Current Chemotherapy. vol. 1, edited by W. Siegenthaler and R. Luthy, pp. 332-334. (Proc. 10th Int. Congr. Chemother., Zurich). Am. Soc. Microbiol. Washington, D.C.

Odelola, H.A. (1978): Antiviral activity of virazole on replication of viruses isolated in Nigeria. In: Current Chemotherapy. vol. 1, edited by W. Siegenthaler and R. Luthy, pp. 334-335. (Proc. 10th Int. Congr. Chemother., Zurich). Am. Soc. Microbiol. Washington, D.C.

Oxford, J.S. (1975): Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribavirin). J. Gen. Virol. 28: 409-414.

Oxford, J.S. (1976): Effects of 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide on influenza virus replication and polypeptide synthesis. J. Antimicrob. Chemother. 1 (Suppl.): 71-76.

Patterson, S. and Macnaughton, M.R. (1981): The distribution of human coronavirus strain 229E on the surface of human diploid cells. J. Gen. Virol. 53: 267-273.

Potter, C.W., Phair, J.P., Vodinelich, L., Fenton, R. and Jennings, R. (1976): Antiviral, immunosuppressive and antitumor effects of ribavirin. Nature. 259: 496-497.

Riski, H. and Hovi, T. (1980): Coronavirus infections of man associated with diseases other than the common cold. J. Med. Virol. 6: 259-265.

Robb, J.A. and Bond, C.W. (1979): Coronaviridae. In: Comprehensive Virology, vol. 14, edited by H. Fraenkel-Conrat and R.R. Wagner, pp. 193-247. Plenum Press, New York.

Sanchez, F.A., Sosa, I.R.G., Vargas, G.M. and Nunez, E.A. (1984): Treatment of type A hepatitis with ribavirin. In: Clinical Applications of Ribavirin, edited by R.A. Smith, V. Knight, and J.A.D. Smith, pp. 193-201. Academic Press, New York.

Sarver, N. and Stollar, V. (1978): Virazole prevents production of Sindbis virus and virus induced cytopathic effect in Aedes albopictus cells. Virol. 91: 267-282.

Scheffler, P., Haghchenas, D. and Wigand, R. (1975): The effect of purine and pyrimidine analogues and virazole on adenovirus replication. Acta Virol. 19: 106-115.

Schmidt, O.W. and Kenny, G.E. (1981): Immunogenicity and antigenicity of human coronaviruses 229E and OC43. Infect. Immun. 32: 1000-1006.

Shannon, W.M. (1977): Selective inhibition of RNA tumor virus replication in vitro and evaluation of candidate antiviral agents in vivo. Ann. N. Y. Acad. Sci. 284: 472-507.

Shannon, W.M. (1984): Mechanisms of action and pharmacology: chemical agents. In: Antiviral Agents and Viral Diseases of Man, edited by G.J. Galasso, T.C. Merigan, and R.A. Buchanan, pp. 55-121. Raven Press, New York.

Siddell, S.G., Anderson, R., Cavanagh, D., Fujiwara, K., Klenk, H.D., Macnaughton, M.R., Pensaert, M., Stohlman, L. and van der Zeijst, B.A.M. (1983): Coronaviridae. Intervirol. 20: 181-189.

Sidwell, R.W. (1980): Ribavirin, in vitro antiviral activity. In: Ribavirin: a Broad Spectrum Antiviral Agent, edited by R.A. Smith, and W. Kirkpatrick, pp. 23-42. Academic Press, New York.

Sidwell, R.W., Allen, L.B., Khare, G.P., Huffman, J.H., Witkowski, J.T., Simon, L.N. and Robins, R.K. (1973): Effect of 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (Virazole, ICN 1229) on herpes and vaccinia keratitis and encephalitis in laboratory animals. Antimicrob. Agents Chemother. 3: 242-246.

Sidwell, R.W., Ellenhorn, M.J., Hillyard, I.W. and Lima, J.M. (1977): Safety evaluations of the broad-spectrum antiviral agent ribavirin (Virazole). In: Current Chemotherpy. vol. 1, edited by W. Siegenthaler, and R. Luthy, pp. 335-338. (Proc. 10th Int. Cong. Chemother., Zuich). Am. Soc. Microbiol. Washington, D.C.

Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T., Simon, L.N. and Robins, R.K. (1972): Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide. Science. 177: 705-706.

Sidwell, R.W., Khare, G.P., Allen, L.B., Huffman, J.H., Witkowski, J.T., Simon, L.N. and Robins, R.K. (1975): In vitro and in vivo effect of 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) on types 1 and 3 parainfluenza virus infections. Chemotherapy. 21: 205-220.

Sidwell, R.W., Robins, R.K. and Hillyard, I.W. (1979): Ribavirin: an antiviral agent. Pharmacol. Ther. 6: 123-146.

Sidwell, R.W., Simon, L.N., Witkowski, J.T. and Robins, R.K. (1974): Antiviral activity of Virazole: review and structure activity relationships. In: Progress in Chemotherapy, vol. II, edited by G.K. Daikos, pp. 889-903. (Proc. 8th Int. Cong. Chemother., Athens)

Sidwell, R.W. and Witkowski, J.T. (1979): Antiviral agents. In: Burger's Medicinal Chemistry, edited by M.E. Wolff, pp. 543-593. Wiley, New York.

Smith, C.M., Fontenelle, L.J., Unger, H., Brox, L.W. and Henderson, J.F. (1974): Inhibition of inosinate dehydrogenase activity in Ehrlich ascites tumor cells in vitro. Biochem. Pharmacol. 23: 2727-2735.

Stohlman, S.A., Sakaguchi, A.Y. and Weiner, L.P. (1979): Characterization of the cold sensitive murine hepatitis virus mutants rescued from latently infected cells by cell fusion. Virol. 98: 448-455.

Streeter, D.G., Simon, L.N., Robins, R.K. and Miller, J.P. (1974): The phosphorylation of ribavirin by deoxyadenosine kinase from rat liver. Differentiation between adenosine and deoxyadenosine kinase. Biochem. 13: 4543-4549.

Streeter, D.G., Witkowski, J.T., Khare, G.P., Sidwell, R.W., Bauer, R.J., Robins, R.K. and Simon, L.N. (1973): Mechanism of action of 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc. Natl. Acad. Sci. U.S.A. 70: 1174-1178.

Sturman, L.S. and Holmes, K.V. (1983): The molecular biology of coronaviruses. Adv. Virus Res. 28: 35-112.

Sturman, L.S. and Takemoto, K.K. (1972): Enhanced growth of a murine coronavirus in transformed mouse cells. Infect. Immun. 6: 501-507.

Tanaka, R., Iwasaki, Y. and Koprowski, H. (1976): Intracisternal virus-like particles in brain of a multiple sclerosis patient. J. Neurol. Sci. 28: 121-126.

Togo, Y. (1973): In vitro effect of virazole against influenza viruses. Antimicrob. Agents Chemother. 4: 641-642.

Tyrrell, D.A.J., Almeida, J.D., Berry, D.M., Cunningham, C.H., Hamre, D., Hofstad, M.S., Malluci, L. and McIntosh, K. (1968): Coronaviruses. Nature. 220: 650.

Tyrrell, D.A.J. and Bynoe, M.L. (1965): Cultivation of a novel type of common-cold virus in organ cultures. Br. Med. J. 1: 1467-1470.

Wege, H., Siddell, S. and ter Meulen, V. (1982): The biology and pathogenesis of coronaviruses. Curr. Top. Microbiol. Immunol. 99: 165-200.

Witkowski, J.T., Robins, R.K., Sidwell, R.W. and Simon, L.N. (1972): Design, synthesis, and broad spectrum antiviral activity of 1-beta-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide and related nucleosides. J. Med. Chem. 15: 1150-1154.

Yoshikura, H. and Tejina, S. (1981): Role of protease in mouse hepatitis virus-induced cell fusion. Virol. 113: 503-511.

APPENDIX

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Extra Tables

LIST OF ABBREVIATIONS

HCV	human coronavirus
HCV/229E	human coronavirus strain 229E
HCV/VH	persistent strain of HCV/229E
HV cells	L132 cells persistently infected with HCV/229E
L132 cells	the human embryonic lung cells
MEM	minimum essential medium
MOI	multiplicity of infection
PBS	phosphate-buffered saline
PFU/mL	plaque forming units per millilitre
SNF	supernatant fluid
ug/mL	microgram per millilitre

Formulation of agar overlay #3 used in this study.

1.8% Oxoid Agar in distilled H_2O*	200 mL
Stock Solution	
Medium 199 (5x)	120 mL
Water sterile	240 mL
Sodium bicarbonate (5.6%)	21.4 mL
BUDR (5-bromodeoxyuridine, 1%)	3 mL
DEAE (diethylamino-ethyl)-dextran (4%)	3 mL
Fetal calf serum	12 mL
Antibiotics (P.S.N.)	0.6 mL
Final volume	600 mL

* 1.8% Agar Bacteriological (Agar No. 1; Oxoid Ltd, Hampshire, England) is prepared, autoclaved, aliquoted and stored at 4°C for use as required. For preparation of overlay #3, 1.8% agar is melted; sterile stock solution containing the remaining ingredients is prepared and added to the agar at a ratio of 2:1 (solution : agar).

Table 9A. Effect of ribavirin on human coronavirus (229E) acute infection after 48 h. at 33 °C*.

RIBAVIRIN ug/mL	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
	PFU/mL x 10^5	PFU/mL x 10^5	PFU/cell	PFU/mL x 10^5	PFU/cell
0	3.0	45.3	1.76	48.3	1.87
10	4.1 (-50.6±94.9)**	16.8 (64.6±11.1)	0.59 (65.4±14.1)	21.0 (56.9±7.4)	0.75 (59.9±4.8)
20	2.7 (12.4±29.2)	11.5 (74.4±4.8)	0.42 (76.1±1.3)	14.2 (70.4±2.5)	0.52 (71.4±3.1)
30	2.0 (31.0±9.5)	7.0 (84.1±0.2)	0.25 (86.2±2.9)	9.0 (81.1±1.1)	0.31 (83.1±3.7)
50	1.01 (64.7±6.7)	3.3 (93.2±5.2)	0.12 (94.0±3.9)	4.3 (91.4±4.8)	0.18 (92.3±3.4)
100	0.8 (77.5±17.5)	0.87 (98.5±0.4)	0.03 (98.2±0.6)	1.7 (96.7±1.7)	0.056 (96.6±2.5)

* Average results of 3 experiments.
** Mean of percent inhibition ± S.D.

Table 10A. Effect of ribavirin on human coronavirus (VH) acute infection after 48 h at 33°C.*

RIBAVIRIN ug/mL	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
	PFU/mL x 10^7	PFU/mL x 10^7	PFU/cell	PFU/mL x 10^7	PFU/cell
0	3.1	6.6	44.8	9.7	59.8
10	3.8 (-11.3±23.3)**	5.7 (22.6±23.9)	39.2 (19.1±17.2)	9.5 (5.1±9.9)	61.0 (-6.9±44.7)
20	2.2 (26.9±9.3)	2.9 (45.8±24.3)	18.0 (39.9±38.3)	5.1 (42.4±21.9)	29.2 (36.7±37.3)
30	0.76 (62.5±22.3)	2.1 (66.5±18.4)	10.7 (67.9±22.1)	2.86 (71.6±2.5)	14.9 (73.9±4.6)
50	0.27 (98.4±5.3)	0.76 (86.0±17.5)	3.5 (83.5±23.7)	1.03 (90.4±7.3)	4.8 (89.8±10.9)
100	0.21 (92.5±3.2)	0.23 (96.5±0.8)	0.89 (98.4± 1.3)	0.44 (95.7±0.9)	2.2 (95.8±2.1)

* Average results of three experiments.
** Mean of percent inhibition ± S.D.

Table 11A: Effect of ribavirin on human coronavirus persistent infection (HV) after 48 h. at 37°C.*

RIBAVIRIN	RELEASED VIRUS	CELL BOUND VIRUS		TOTAL VIRUS	
ug/mL	PFU/mL x 10^4	PFU/mL x 10^4	PFU/cell	PFU/mL x 10^4	PFU/cell
0	12.91	67.45	0.19	80.36	0.27
10	1.96 (84.0±2.2)**	27.13 (40.1±41.1)	0.11 (31.0±44.1)	20.09 (57.5±30.4)	0.13 (50.7±34.4)
30	0.59 (95.3±1.9)	14.92 (84.5±9.0)	0.045 (80.1±9.9)	15.51 (86.7±9.2)	0.049 (83.4±10.3)
50	0.42 (96.7±2.9)	2.35 (94.7±2.6)	0.013 (91.3±4.7)	2.77 (96.2±1.3)	0.016 (93.7±2.3)
70	0.34 (97.2±2.2)	0.97 (97.7±1.6)	0.0055 (95.7±3.6)	1.31 (98.1±0.8)	0.0084 (96.7±1.8)
100	0.32 (97.4±2.9)	0.83 (99.1±0.4)	0.0035 (98.5±0.9)	1.15 (98.8±0.6)	0.0056 (98.1±1.0)

* Average results of 3 experiments.
** Mean of percent inhibition ± S.D.

Table 12A. The inhibition of HCV/229E plaques by ribavirin.

229E INOCULUM PFU/mL \ RIBAVIRIN ug/mL	NUMBER OF PLAQUES*						
	0	1	10	20	30	50	70
$8.1x10^4$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	3**
8100	TNTC	TNTC	TNTC	TNTC	TNTC	28**	0
810	TNTC	TNTC	TNTC	TNTC	TNTC	11.5**	8**
81	17.25	18.25	14.75	13.0	6.25	0.5**	0
8.1	1.5	3.5	1.0	1.75	1.0	0	0

* Average numbers of plaques/well of two experiments (4 wells). Underlined data indicate results available from one experiment only.
** Small and indistinct plaques oberved.

Table 13A. The inhibition of HCV/VH plaques by ribavirin

VH INOCULUM PFU/mL \ RIBAVIRIN ug/mL	NUMBER OF PLAQUES*						
	0	1	10	20	30	50	70
$2.8x10^5$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
$2.8X10^4$	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	13**
2800	TNTC	TNTC	TNTC	TNTC	TNTC	5.5**	2.5**
280	42.3	37.5	39.5	34.5	33.5	2.8**	0
28	5.0	3.3	4.3	0	0	0	0
2.8	0.5	0.25	0.5	0.25	0	0	0

* Average numbers of plaques/well of two experiments (4wells). Underlined data indicate results available from one experiment only.
** Small and indistinct plaques observed.

Table 16A. The effect of guanosine on the antiviral activity of ribavirin on 229E and VH virus plaque formation.

RIBAVIRIN	GUANOSINE	NUMBER OF PLAQUES*			
50 ug/mL	200 ug/mL	229E	% Change	VH	% Change
-	-	16.2±6.2	0	11.3±2.5	0
+	-	0	-100	0	-100
-	+	12.5±3.4	- 22.8	7.3±3.8	- 35.4
+	+	9.5±1.9**	- 41.4	8.7±3.1**	- 23.0

* Average of 3 experiments. Innocula for both 229E and VH ranged between 40-70 PFU/mL.

** Small plaques observed.

Table 20. Effect of ribavirin on total PFU/cell production during the curing experiment.

RIBAVIRIN	CELL PASSAGE NUMBER										
ug/mL	1	2	3	4	5	6	7	8	9	10	11
0	0.13*	0.09	0.07	0.12	(*)	(*)	(*)	0.17	0.44	0.43	0.31
10	0.049	0.007	0.007	0.036	0.043	0.062	0.19	0.19	0.19	0.17	0.25
50	0.027	7×10^{-6}	0.0	0.0	0.0	0.0001	0.0003	0.28**	0.68	0.46	0.45

* Total PFU/cell.
(*) No data available because of sample contamination.
** Drug removed from this cell passage.