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Molecular Aspects of the Antiviral Activity of Ribavirin on Venezuelan Equine Encephalomyelitis Virus

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Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximide) is a nucleoside analog having broad-spectrum antiviral activity against both DNA and RNA viruses (R. W. Sidwell, R. K. Robins, and I. W. Hillyard, Pharmacol. Ther., in press). A variety of specific effects on host cell metabolism have been attributed to ribavirin or its metabolites. Ribavirin monophosphate appears to be a competitive inhibitor of inosine monophosphate dehydrogenase activity and GMP synthesis, whereas ribavirin triphosphate is a selective inhibitor of influenza virus RNA polymerase. Additional effects of ribavirin include inhibition of thymidine phosphorylation and synthesis of DNA, RNA, and proteins (R. A. Smith, R. W. Sidwell, and R. K. Robins, Annu. Rev. Pharmacol., in press). Contrasting reports have been published which contradict many of these alleged cellular effects (1). The pharmacological mechanism of action of ribavirin thus remains obscure.

In an attempt to clarify the mode and specificity of action of ribavirin, we examined its effects on cellular metabolism and on the replication of Venezuelan equine encephalomyelitis (VEE) virus grown in BHK-21 cells as a model system.

VEE virus in this system rapidly replicates and yields a 3-log increase in virions within 5 h after infection at a multiplicity of inoculum of about two virus particles per cell. A 4-log increase in virus titer is found at 17 h. Ribavirin at concentrations as low as 10 μg/ml inhibits virus replication by more than 99% compared with nontreated cultures.

The uptake of radiolabeled precursors into trichloroacetic acid-soluble and -insoluble material during a 30-min pulse was then examined in control and infected cells pretreated for 5 h with ribavirin (25 to 800 μg/ml). Ribavirin inhibited both trichloroacetic acid-soluble and -insoluble uptake of uridine by control and infected cells. This inhibition was reversed at higher concentrations. Guanosine uptake was enhanced at low doses. Incorporation into trichloroacetic acid-insoluble material was inhibited by about 35% at 100 μg of ribavirin per ml.

The complex nature of these results is not readily explainable. Results suggest that ribavirin may alter cellular nucleotide pools and cause significant changes in the specific activity of isotopic labeling. Inhibition of inosine uptake and incorporation by low doses of ribavirin contributes to altered pool sizes. Inosine utilization was inhibited by approximately 85% at the lowest dose of ribavirin tested (25 μg/ml). This observation is consistent with the inhibition of inosine monophosphate dehydrogenase by ribavirin and a subsequent change in the cellular guanosine pool. However, the effect of ribavirin on inosine monophosphate dehydrogenase is not thought to be a principal site of antiviral action. We were led to this proposal because inosine utilization in virus-infected cells is decreased by more than 80% compared with uninfected cultures. Doses of ribavirin which reduce virion production by 99% have only a minimal additional inhibitory effect on inosine incorporation.

Inhibition of nucleotide incorporation in ribavirin-treated cells could result from the inhibition of RNA polymerase. Therefore, we determined the rate of [^{3}H]UTP incorporation into BHK-21 cells made permeable to large, charged molecules by exposure to lysolecithin (200 μg/ml) at 4°C. This approach was used to determine the effects of ribavirin and its phosphorylated derivatives on RNA synthesis; little inhibitory effect was observed (Fig. 1A). These observations suggest that neither ribavirin nor its phosphorylated derivatives inhibit RNA polymerase activity in VEE-infected BHK-21 cells.

[^{3}H]uridine-labeled mRNA from control and infected cells treated with varying concentra-

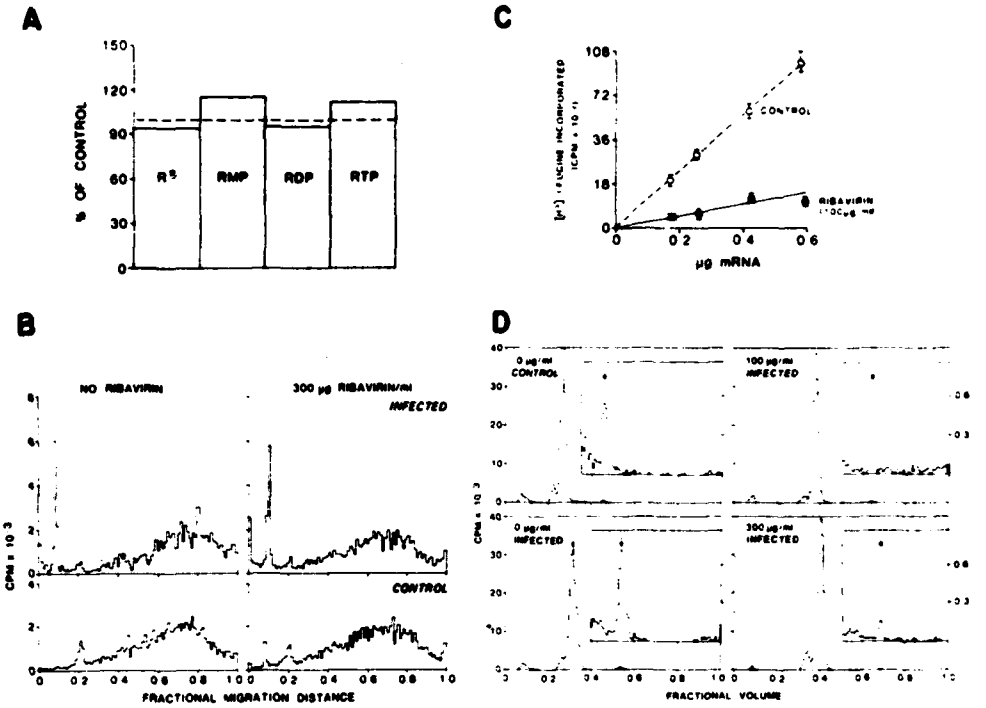

FIG. 1. *Effects of ribavirin on cellular and viral RNA. (A) Effects of ribavirin (R) and phosphorylated derivatives (RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate) on [^{3}H]UTP incorporation in permeable VEE-infected BHK-21 cells; (B) polyacrylamide gel electrophoresis analysis of mRNA isolated from BHK-21 cells; (C) in vitro translation of mRNA isolated from ribavirin-treated and untreated VEE-infected cells; (D) diethylaminoethyl-cellulose chromatography of mRNA digests.*

tions (10 to 300 μg/ml) of ribavirin was then isolated on oligodeoxythymidine columns and subfractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The 42S viral RNA genome is readily recognized as a single peak at a relative migration distance of about 0.1 and represents 10 to 11% of total mRNA (Fig. 1B). Treatment of infected cell cultures with 300 μg of ribavirin per ml did not diminish the relative quantity of viral mRNA. These observations indicate that ribavirin has no specific inhibitory effects on the synthesis of viral genome.

Since interference with synthesis of viral genetic information is not a probable mechanism for ribavirin's antiviral activity, we examined the capacity of mRNA from treated cells to direct the synthesis of proteins in an in vitro, rabbit reticulocyte, cell-free translation system. mRNA from infected cells treated with 100 μg of ribavirin per ml was significantly less efficient than that from untreated, infected cells in its ability to direct the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material (Fig. 1C).

Recently, Goswami et al. (2) reported that ribavirin triphosphate is a potent inhibitor of the 5'-terminal guanylation of vaccinia mRNA. Since the 5'-terminal 7-methyl guanine in mRNA appears to be required for efficient translation (3), we studied the effects of ribavirin on the capping of mRNA in our model system. [^{3}H]guanine-labeled mRNA was isolated on oligodeoxythymidine columns and digested with RNase. The resulting mononucleotides were chromatographed on diethylaminoethyl-cellulose, using a linear NaCl gradient. A minor radioactive peak eluting after the major mononucleotide peak and corresponding to the elution profile of a 7mGppp "cap" standard was found in all chromatograms of mRNA digests from control and VEE-infected cells (Fig. 1D). The area under the cap peak which was resolved from mRNA preparation isolated from virus-infected cells treated with ribavirin at concentrations of 50 to 300 μg/ml was reduced nearly tenfold.

We conclude, therefore, that the pharmacological mechanism of action of ribavirin is not specifically antiviral. We propose that ribavirin

does not inhibit viral transcription but interferes with translation of viral messenger by replacing or inhibiting formation of the cap structure on the viral RNA genome.

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