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Mechanism of La Crosse Virus Inhibition by Ribavirin

LISA F. CASSIDY† AND JEAN L. PATTERSON*

Division of Infectious Diseases, Children's Hospital, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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The effect of ribavirin on the growth and replication of La Crosse virus was examined. The data suggest that low concentrations of ribavirin have a marked effect on the initial steps of La Crosse virus transcription. The therapeutic potential of ribavirin in the treatment of human California encephalitis serotype infections is discussed in light of these findings.

La Crosse (LAC) virus is a member of the California encephalitis serogroup of bunyaviruses (15). It was initially isolated in 1964 from the frozen brain tissue of a 4-year-old female who died from meningoencephalitis in La Crosse, Wisconsin (22). The virus is transmitted in nature through the bite of infected mosquitoes. It is not pathogenic to insect cells (17). The genome of the LAC virus consists of three segments of negative-strand RNA, labeled small (S), medium (M), and large (L), each contained within a separate nucleocapsid (3, 11). These nucleocapsids serve as templates for mRNA synthesis, initiating transcription by a cap-snatching mechanism similar to that of influenza virus (9, 13). Like other negative-strand viruses, LAC virus replicates through the synthesis of a full-length plus-stranded intermediate, the antigenome.

We examined the effects of the broad-spectrum antiviral agent ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) on LAC virus infection. This drug possesses broad antiviral activity (4, 20), but its molecular mode of action remains controversial. Our studies examined the effect of ribavirin on several stages of LAC virus replication.

A plaque reduction assay was used initially to measure the activity of ribavirin against LAC virus. Monolayers of BHK cells were infected with LAC virus. After a 0.5-h absorption period, the inoculum was removed, and agar overlays containing 0, 10, 30, and 50 μg of ribavirin per ml were added. After 4 days, the cells were stained with 0.1% neutral red and the number of PFU was determined. The initial stock of virus added to all the wells had a titer of 8.4 PFU/ml, and when no ribavirin was added an average of 8 PFU/ml was recovered. When 10 μg/ml was added, one-half of the viral progeny could be recovered. Cell cultures overlaid with medium containing 30 or 50 μg of ribavirin per ml showed complete inhibition of plaque formation. In previous cases in which the activity of ribavirin against other negative-stranded RNA viruses was tested, 100 and 200 μg of ribavirin per ml was required for total supression of viral progeny (21, 23). Since as little as 30 μg of ribavirin per ml completely inhibits LAC virus plaque formation, LAC is relatively susceptible to inhibition by this drug. These data support the findings of Sidwell et al. (19) that similar concentrations of ribavirin are effective in inhibiting two strains of Punta Toro virus, another bunyavirus.

We attempted to determine whether the block in replication of the virus was at the level of mRNA transcription. S-mRNA synthesis was monitored as an indicator of transcriptional efficiency. Patterson et al. (13) reported that La Crosse virions contain an RNA-dependent RNA polymerase which cleaves the host cell mRNA 11 to 15 nucleotides from the 5′ methylated cap and uses this cleavage product as a primer during transcription of viral mRNAs. To first determine whether this priming event was inhibited upon addition of ribavirin, total cellular mRNA (CsCl pellet RNA) and nucleocapsid RNA were isolated from cytoplasmic extracts by using CsCl gradient-isolated fractions from uninfected and LAC virus-infected cells (10). S-mRNA was evaluated by primer extension analysis as described previously (13, 14, 18). Primer extension products represent extensions of mRNAs that have 50 bases of negative genome-encoded message plus an 11- to 15-base nontemplated 5′ extension derived from precapped host cell mRNA (13). Figure 1A, lane 2, shows the primer extension analysis of mRNA from LAC virus-infected BHK cells. When the concentration of ribavirin added to the host cells during transcription was increased from 10 to 30 μg/ml (lanes 4 and 5), there was a 5-unit drop in the relative area of the S-mRNA (20% of the total scale). No further decrease was observed when the concentration of ribavirin was raised from 30 to 50 μg/ml. At the highest concentration of RNA (lane 2), it was difficult to determine whether there was excess primer. Therefore, the concentrations of S-mRNA synthesized in the presence of any concentration of ribavirin are the most reliable values, because the lower concentrations of RNA were measured.

The addition of ribavirin did not seem to change the primer, since the length and appearance of the 5′ cap remained the same. There was, however, a significant quantitative decrease in the total amount of S-mRNA produced.

In order to demonstrate that the polymerase continues beyond this position, the mRNAs were examined by dot blot analysis. Increasing concentrations of ribavirin, from 10 to 30 μg/ml, inhibited mRNA synthesis by 6 units of relative area (20% of total scale) (Fig. 2). When the concentration of ribavirin was raised from 30 to 50 μg/ml, there was little change in the relative area. The inhibition found from 10 to 50 μg of ribavirin per ml was similar to that seen at the 5′ end of this transcript. These results make the point that the inhibition is the same at the 5′ end as that seen further downstream, suggesting that the inhibition occurs at initiation of transcription.

Stable antigenome RNAs of LAC virus are exclusively found in nucleocapsid structures. To determine whether the presence of ribavirin would affect antigenome synthesis, we isolated nucleocapsid structures from LAC virus-infected

* Corresponding author.

† Present address: Department of Immunology and Medical Microbiology, College of Medicine, University of Florida, Gainesville, FL 32610.

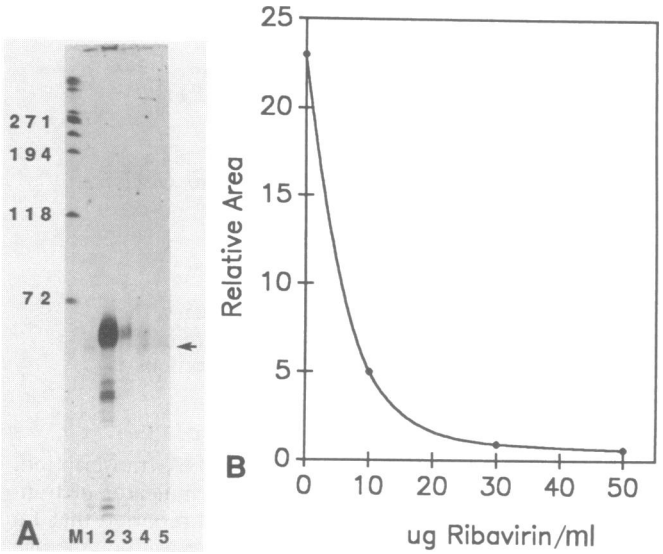

FIG. 1. Effect of ribavirin on primer generation and initiation of LAC virus S-mRNA transcription. Cells were infected at a multiplicity of infection of 1.4 PFU/ml as described for the analysis of transcriptional events for LAC virus (16). CsCl pellet RNAs were isolated at 6 h postinfection (before any cytopathic effect) from LAC virus-infected BHK cells that had been treated with 0 (lane 2), 10 (lane 3), 30 (lane 4), or 50 (lane 5) μg of ribavirin per ml. CsCl pellet RNA from uninfected BHK cells was also isolated (lane 1). (A) A 50-μg portion of each RNA sample was used for primer extension of end-labeled oligodeoxynucleotides as described in the text. The numbers on the left refer to marker positions. These products were approximately 60 to 65 nucleotides in length and were not detected in mRNA from uninfected cells (lane 1). The band seen just below the cap structures (arrowhead) is a host cell product, since it also appears in samples of uninfected extracts (lane 1). (B) Relative area under the peaks obtained when the bands at position 65 were analyzed by densitometry and plotted versus ribavirin concentration.

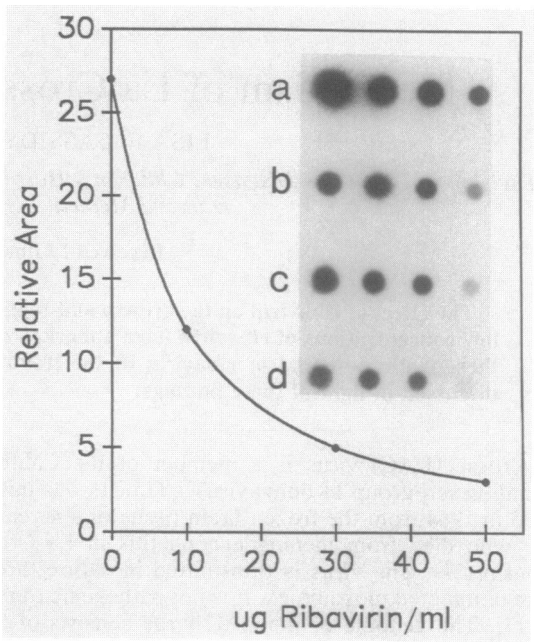

FIG. 2. Effect of ribavirin on elongation of mRNA transcripts. CsCl pellet RNAs were isolated at 6 h postinfection from LAC-infected BHK cells (as described for Fig. 1) that had been treated with 10 (b), 30 (c), or 50 (d) μg of ribavirin per ml. mRNA was also isolated from LAC virus-infected cells that received no drug treatment (a). A 5-μg portion of each mRNA sample was applied to a nylon membrane, along with 1:5, 1:10, and 1:20 dilutions. A riboprobe containing a sequence which is complementary to nucleotides 196 to 764 from nucleotide no. 1 of the LAC virus S-mRNA was synthesized by the method recommended by the manufacturer (Promega Biotec, Madison, Wis.). The probe was used to hybridize with the CsCl pellet RNA from uninfected and LAC virus-infected cells in a dot blot analysis (Zeta-Probe) as described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). To assure that there was excess probe, various dilutions of RNAs were tested. The graph shows the relative area under the peaks obtained when the 1:20 dilutions were analyzed by densitometry and plotted versus ribavirin concentration.

cells (13) that had been treated with ribavirin. When 30 to 50 μg of ribavirin per ml was added (Fig. 3c and d), antigenome production was not detected in the dot blot analysis, although, as shown in Fig. 2, some mRNA could still be detected at these concentrations of ribavirin.

The inhibition of LAC virus production appeared to occur early in the replicative cycle. Antiviral activity was observed within 6 h postinfection, during primary transcription of the mRNA. The dot blot analysis demonstrated that the inhibition of RNA synthesis measured at the 5′ end of the message (Fig. 1) is proportional to the inhibition measured further downstream (Fig. 2). This is consistent with our previous report, which showed that ribavirin cannot be incorporated into the growing chain and causes no further termination of transcription after initiation (24).

The overall scheme for the replication of LAC virus is similar to that used by other negative-strand RNA viruses, such as vesicular stomatitis virus. After primary transcription, nucleocapsid assembly takes place, resulting in the production of a full-length antigenome (10). Continued protein synthesis is a requirement for antigenome production. It might be expected that the inhibition of antigenome synthesis would be directly proportional to the inhibition of mRNA synthesis. However, this is not the case. The addition of ribavirin diminished antigenome production more than it did mRNA synthesis (Fig. 3). Although it cannot be ruled out that there is a direct block on antigenome RNA synthesis, it is more likely that the reduction in the concentration of mRNA results in a lowered amount of viral proteins and, subsequently, in a block in nucleocapsid assembly (1, 2, 8, 16). Insufficient concentrations of the viral proteins and N protein, in particular, are probably the overriding factors in the inhibition of antigenome synthesis. Inhibition of viral progeny is more sensitive to ribavirin than is nucleocapsid inhibition. Indeed, direct linear effects on later stages in viral replication and assembly are difficult to ascertain, since any deregulation of the system at the mRNA level is presumably amplified at all levels of viral progeny production.

The data presented here suggests that ribavirin has a direct effect on LAC virus polymerase activity. Viral replicative enzymes such as RNA-dependent RNA polymerases are often the targets of ribavirin inhibition. Influenza virus (25) and vesicular stomatitis virus (24) polymerases have both been shown to be direct targets of ribavirin. Fernandez-Larsson et al. (6) and Fernandez-Larsson and Patterson (J. Gen. Virol., in press) further demonstrated that the block in vesicular stomatitis virus replication was in the initiation of transcription.

It is most important that the addition of relatively low concentrations of ribavirin inhibited LAC virus irrespective of the technique used to measure the inhibition. The marked susceptibility of LAC virus to ribavirin may be important in

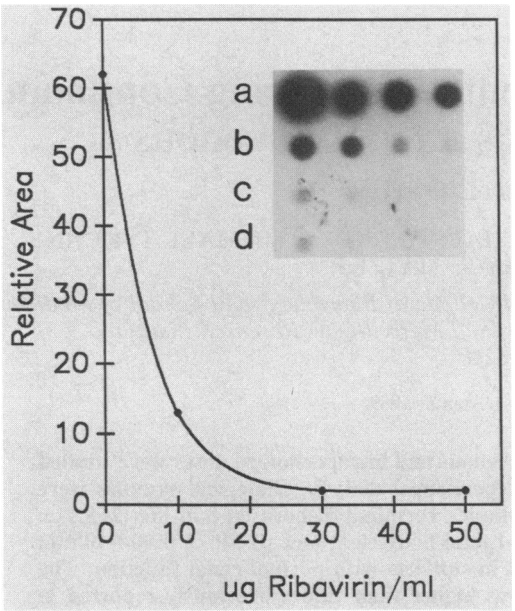

FIG. 3. Effect of ribavirin on antigenome synthesis. Nucleocapsids banded in CsCl were isolated at 6 h postinfection from LAC virus-infected BHK cells (as described for Fig. 1) that had been treated with 10 (b), 30 (c), or 50 (d) μg of ribavirin per ml. Nucleocapsids were also isolated from LAC-infected cells that received no drug treatment (a). After proteinase K digestion, extracted RNA was applied to a nylon membrane along with 1:2, 1:4, and 1:8 dilutions. The presence of viral antigenomes was detected by using riboprobe 196-764. The graph shows the relative area under the peaks obtained when the 1:2 dilutions were analyzed by densitometry and plotted versus ribavirin concentrations.

treating the intracerebral infections that result from LAC virus infection. Ferrara et al. (7) report that ribavirin inefficiently crossed the blood-brain barrier and was unable to accumulate in the brains of animals. However, two groups have subsequently been able to measure a maximum concentration of ribavirin of 9.5 μM in spinal fluid samples of patients receiving high-dose oral therapy (5, 12). Ribavirin may be an important therapeutic agent of treating human LAC virus infections, considering that the level of ribavirin required for in vivo inhibition of LAC virus (0.3 μM) is significantly lower than the concentration found in the cerebrospinal fluid.

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