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# Effect of Ribavirin on Rous Sarcoma Virus Transformation

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Ribavirin inhibited the expression of cellular transformation in normal rat kidney cells transformed by a temperature-sensitive mutant of Rous sarcoma virus and chicken embryo fibroblasts infected with either a temperature-sensitive mutant or wild-type Rous sarcoma virus. Ribavirin also inhibited replication of the Rous sarcoma viruses in chicken embryo fibroblasts. The effect of ribavirin on cellular transformation was not permanent, as removal of the drug resulted in reversion to the transformed phenotype. The concentration of ribavirin necessary to inhibit the expression of cellular transformation was cytostatic for the cell lines used in this study.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleoside that has been reported to have broad-spectrum antiviral activity against deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses, both in vitro and in vivo. Huffman and his co-workers (5) reported an inhibitory effect by ribavirin in vitro against the following viruses: adenovirus type 3, herpes simplex virus types 1 and 2, myxoma, cytomegalovirus, vaccinia, parainfluenza types 1 and 3, Newcastle disease virus, and measles. Inhibition in vivo has been reported by Sidwell et al. (9) against Friend leukemia virus in mice and by Potter et al. (8) against influenza virus in ferrets. Reviews of the antiviral activity of ribavirin have recently been published (10, 11).

A search of the literature reveals no reports on the effect of ribavirin against the morphological expression of cell transformation. In this study, the effect of ribavirin on the expression of cellular transformation in chicken embryo fibroblast (CEF) cells infected with either wild-type or temperature-sensitive mutants of Rous sarcoma virus (RSV) and in normal rat kidney (NRK) cells transformed by a temperature-sensitive mutant of RSV has been investigated.

The cell lines used in this study were primary CEF cells, NRK cells, and cell lines derived after transformation of NRK cells with either temperature-sensitive mutants of RSV (LA-31 or LA-25) or wild-type RSV (B-77).

Primary CEF of c/o phenotype were prepared directly from fertile eggs (SPAFAS, Norwalk, Conn.) according to published techniques (13). All cells used in this study were grown in F-10 (Ham) medium with Hanks balanced salt solution (GIBCO Laboratories, Berkeley, Calif.), supplemented with 5% cadet calf serum, 10% tryptose phosphate broth, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were grown in humidified 5% $CO_2$ incubators.

The following strains of wild-type RSV were used: Prague-A (PR-A), Schmidt-Ruppin-D (SR-D), and Bratislava-77 (B-77). A mutant of the PR-A strain (LA-23) was also used. These viruses were obtained from Peter Vogt's laboratory in California (4).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was obtained from ICN Laboratories, Irvine, Calif., with the help of Lois B. Allen. Dilutions of the drug were made with the culture medium described above.

The effect of ribavirin on the morphological transformation of CEF cells by four different RSV strains is shown in Table 1. CEF cells were infected with $1.2 \times 10^5$ focus-forming units (multiplicity of infection, 0.1) of the different RSV strains. The infected cells were incubated in the presence of varying amounts of ribavirin at either 39°C for the three wild-type RSV strains (PR-A, SR-D, B-77) or at 33°C, the permissive temperature of LA-23. After 6 days, the degree of cell transformation was judged by evaluating the percentage of cell rounding. Ribavirin effectively inhibited the morphological expression of cell transformation at all concentrations tested (Table 1). The supernatants from these infected cells were collected, and a focus assay was performed to determine the effect of ribavirin on virus replication (13). Ribavirin greatly inhibited virus production (Fig. 1). The wild-type strain B-77 showed a slight resistance to ribavirin at 10 μg/ml.

After having determined the effect of ribavirin on morphological transformation and virus production in CEF cells, we turned our attention to

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NRK cells transformed by RSV. We used an NRK cell line transformed by LA-31, a temperature-sensitive mutant of the PR-A strain. This cell line exhibits a transformed phenotype at the permissive temperature (33°C) and reverts to a normal fibroblast phenotype at the nonpermissive temperature (39°C). In addition to the round transformed phenotype exhibited at 33°C, these cells grow to a high saturation density but do not produce virus particles. By shifting the temperature, one can control the morphological expression of transformation (3).

TABLE 1. *Effect of ribavirin on morphological transformation of CEF by RSV*

| Virus | Dose of ribavirin (μg/ml)[a] | Cell rounding (%)[b] |
|---|---|---|
| LA-23 | 0 | 40 |
| | 10 | 0 |
| | 50 | 0 |
| | 75 | 0 |
| | 100 | 0 |
| PR-A | 0 | 90 |
| | 10 | 0 |
| | 50 | 0 |
| | 75 | 0 |
| | 100 | 0 |
| B-77 | 0 | 80 |
| | 10 | 10 |
| | 50 | 0 |
| | 75 | 0 |
| | 100 | 0 |
| SR-D | 0 | 90 |
| | 10 | 0 |
| | 50 | 0 |
| | 75 | 0 |
| | 100 | 0 |

[a] Ribavirin was added to the cultures at the time of virus inoculation.

[b] Cell cultures were evaluated 6 days after infection with the viruses.

The effect of ribavirin on the maintenance of morphological transformation in the LA-31-NRK cell line grown at the permissive temperature (33°C) is shown in Fig. 2. The cells were grown in 60-mm plates at 33°C until the majority of the cultures exhibited greater than 90% cell rounding. Ribavirin was then added, and the cultures were examined daily for 6 days, with the degree of cell rounding being noted. As the concentration of ribavirin increased, the degree of cell rounding decreased. At a concentration of 100 μg/ml, the cells showed a 60% decrease in cell rounding. If the cultures were trypsinized, replated in 60-mm plates, and fed fresh medium without ribavirin, the cells would revert to the transformed phenotype at levels comparable to those of the controls within 1 week (Fig. 3).

The LA-31-NRK transformed cells were then grown at the nonpermissive temperature (39°C) until all cultures showed 20% or less cell rounding. Ribavirin was added to the medium, and the cultures were shifted to the permissive temperature (33°C). The cultures were examined daily for 6 days, with the degree of cell rounding being noted. The results of the shift from the nonpermissive to the permissive temperature are shown in Fig. 4. At all concentrations tested,

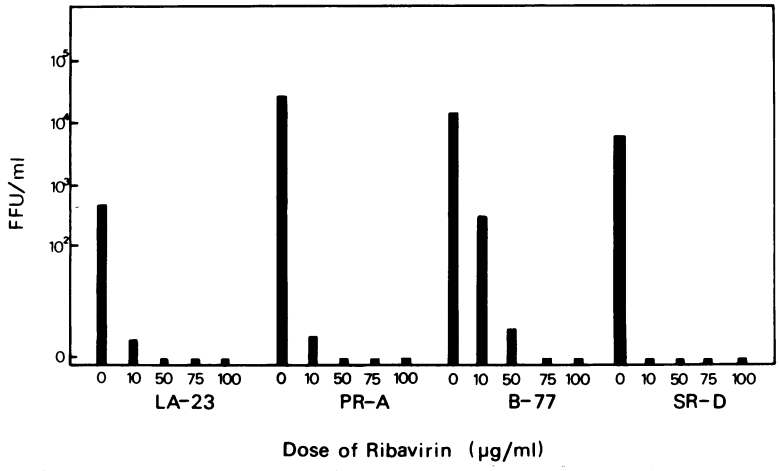


FIG. 1. *Effect of ribavirin on replication of RSV. Ribavirin was added to the cultures at the time of virus inoculation, and new medium also containing ribavirin was refed to the cultures after 3 days. Focus-forming units (FFU) per milliliter were determined by serial dilution of virus supernatants from 6-day-old infected cultures on CEF.*

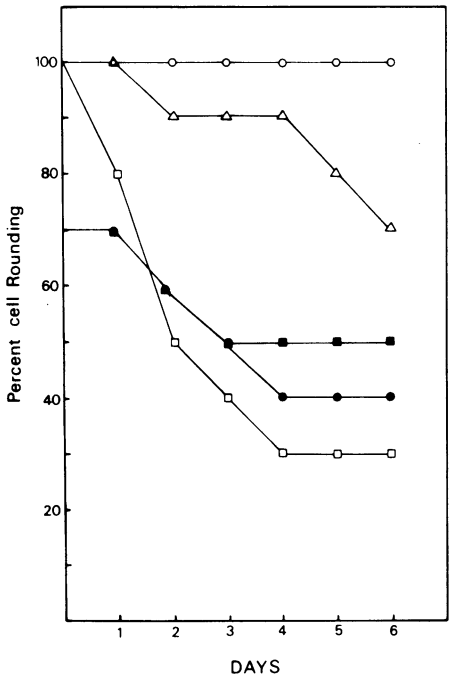


FIG. 2. *Effect of ribavirin on the maintenance of the transformed phenotype of RSV-transformed rabbit kidney cells. Ribavirin-containing or control medium was added to confluent transformed cell cultures on day 0, and the cultures were refed on day 3. The total percent cell rounding was judged for each culture daily by examination with a light microscope. Points represent two to three cultures per drug dose. Symbols: ○, 0 μg/ml; △, 10 μg/ml; ■, 50 μg/ml; ●, 75 μg/ml; □, 100 μg/ml.*

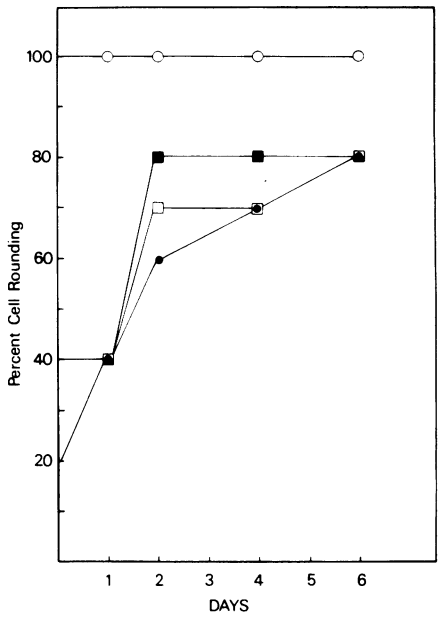


FIG. 3. *Reversion to the transformed phenotype after removal of ribavirin. RSV-transformed rabbit kidney cells were grown at the permissive temperature in the presence of ribavirin until the majority of the cultures exhibited the normal phenotype. The cultures were then trypsinized on day 0 and replated in 60-mm plates in the absence of ribavirin. The cultures were refed with fresh medium on day 4. Total percent cell rounding was judged for each culture daily by examination with a light microscope. Points represent two to three cultures per drug dose. Symbols: ○, 0 μg/ml; ■, 50 μg/ml; ●, 75 μg/ml; □, 100 μg/ml.*

ribavirin completely inhibited the reversion from the normal to transformed phenotype.

To determine whether ribavirin had any effect on the growth of these cell lines, growth curves were determined by using different concentrations of ribavirin. The cells were seeded directly into medium containing ribavirin and grown in the presence of the drug. The cells from three plates per drug dose were counted daily with a Hycell counter, and the cell counts were averaged together. At a concentration of 10 μg/ml, ribavirin had only a slight effect on the cell growth of the three cell lines, with the most marked difference being for the B-77-transformed cell line (Fig. 5). At concentrations greater than 10 μg/ml, ribavirin appeared to stop cell division. Because removal of ribavirin after 6 days of treatment at 33°C of the LA-31-NRK cells allows return to the transformed phenotype at levels equal to those of the controls, we feel that ribavirin is not killing large numbers of cells even though cell division is retarded.

Kawai and Hanafusa (6) and Ash et al. (1) have shown that protein synthesis inhibitors, such as cycloheximide and puromycin, caused a reversion from the transformed phenotype to normal phenotype in cells infected with a temperature-sensitive RSV. In addition, actinomycin D, an RNA synthesis inhibitor, has also been found to cause this morphological reversion (Y. C. Chen, unpublished data). These results would indicate that virus-directed transcription and translation are necessary for the maintenance of the transformed state. In class T-1 mutants (LA-23 and LA-31), the *src* gene product is irreversibly inactivated at the nonpermissive temperature and needs to be resynthesized upon the shift to the permissive temperature to express the transformed phenotype (14). Previous papers by Streeter et al. (12), Lowe et al. (7), and Browne (2) indicate that one mechanism of action of ribavirin is the inhibition of RNA synthesis by depletion of the cellular purine nucleotide pools. The monophosphate metabolite of

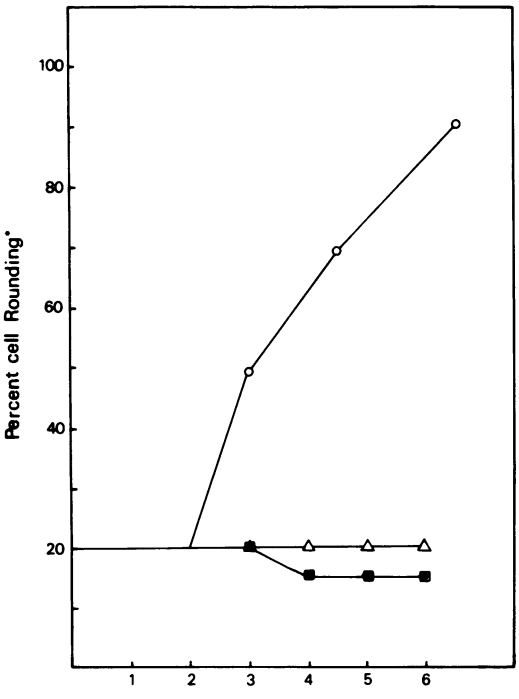


FIG. 4. *Effect of ribavirin on the reversion to the transformed phenotype. RSV-transformed rabbit kidney cells were grown at the nonpermissive temperature (39° C) until the majority of the cultures exhibited the normal phenotype. Ribavirin-containing or control medium was added to the cultures, and the cultures were shifted to the permissive temperature (33° C) at time zero. The cultures were refed with ribavirin on day 3. Total percent cell rounding was judged for each culture daily by examination with a light microscope. Points represent 2 to 3 cultures per drug dose. Symbols: ○, 0 μg/ml; △, 10 μg/ml; ●, 75 μg/ml; □, 100 μg/ml.*

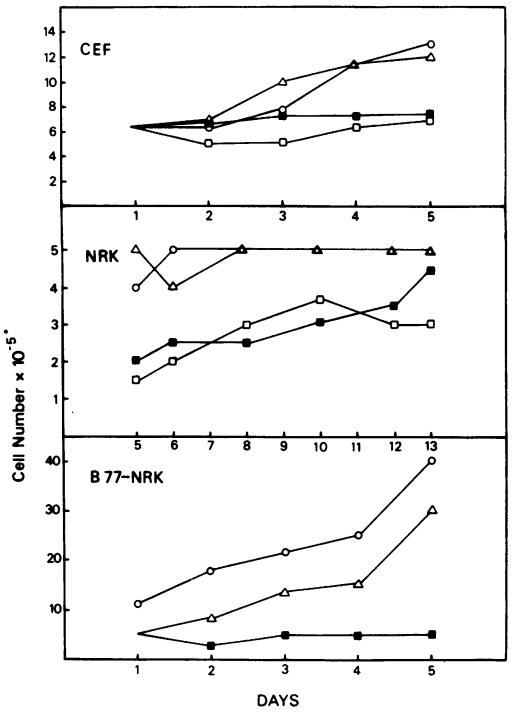


FIG. 5. *Effect of ribavirin on cell growth of CEF, NRK, and B-77-transformed NRK cell lines. Cells were seeded in medium containing ribavirin at day 0 and refed with medium containing ribavirin every third day. Cell numbers were determined by counting trypsinized cells in a Hycell counter. Points represent an average of three cultures per drug dose. Symbols: ○, 0 μg/ml; △, 10 μg/ml; ■, 50 μg/ml; ●, 75 μg/ml; □, 100 μg/ml.*

ribavirin specifically inhibits the normal host cell enzyme inosine 5'-monophosphate dehydrogenase. In addition, other possible mechanisms of action of ribavirin include the inhibition of viral polymerases by the triphosphate of ribavirin, inhibition of viral polypeptide synthesis, and competition with guanosine in the capping of messenger RNA (11). All of these mechanisms would result in the inhibition of either cellular or virus-coded RNA synthesis, which would effectively explain the loss of transformed phenotype and inhibition of virus production shown in this study. It would also explain the cytostatic effect on cellular growth.

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