JOURNAL OF VIROLOGY, Jan. 1987, p. 233–235
0022-538X/87/010233-03$02.00/0

Vol. 61, No. 1

Ribavirin Cures Cells of a Persistent Infection with Foot-and-Mouth Disease Virus In Vitro

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Received 29 April 1986/Accepted 23 September 1986

Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) eliminates foot-and-mouth disease virus from persistently infected cell cultures. The latter are 10-fold more sensitive to ribavirin than lytically infected cells. In treated cells no viral RNA or proteins could be detected by dot-blot hybridization to cDNA probes, virus and RNA infectivity assays, or immunofluorescence. A potential application of the drug for the treatment of animals carrying the virus is suggested.

Foot-and-mouth disease is the most economically important animal viral disease. Its causative agent, foot-and-mouth disease virus (FMDV) is a picornavirus (2, 9) that may produce, in addition to the well-characterized acute infection, a persistent infection both in nonimmunized and in immunized animals (13, 14) by mechanisms that are not understood. We have established BHK-21 cell lines persistently infected with FMDV (4) in the hope that they will aid in the study of the mechanism of FMDV persistence and of FMDV genetic variability (5, 6). For several experiments it was desirable to derive, from the persistently infected cultures, representative cell populations (avoiding cell cloning) free of virus. This was not achieved by treatment of the cells with anti-FMDV antibodies (4). Here we show that the antiviral agent ribavirin (1-β-D-ribofuranosyl-1*H*1,2,4-triazole-3-carboxamide) (8, 11) cures BHK-21 cells from persistent infection with FMDV.

The persistently infected cell lines were established by growth of the cells that survived a standard cytolytic infection with FMDV (4). The cells have been termed C_1-BHK-Rc1 to indicate the FMDV serotype (C_1), resistance to superinfection by FMDV (R), and the initial BHK-21 cell clone used in the establishment of persistence (c1). Procedures for FMDV RNA analysis by dot-blot hybridization to cloned cDNA probes and for indirect immunofluorescence have been described (4). RNA infectivity was quantitated by the following modification of published procedures (1, 12). Total cellular RNA was extracted from cells (4) and fractionated by sucrose gradient sedimentation (15 to 30% sucrose) in 10 mM Tris hydrochloride (pH 7.4)–0.1 M NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate with an SW40 rotor at 38,000 rpm for 5 h at 10°C. RNA from each fraction was recovered by ethanol precipitation and the infectivity was determined as follows. Washed BHK-21 cell monolayers were treated with DEAE-dextran (0.5×10^6 molecular weight) at 800 μg/ml in culture medium adjusted to 50 mM Tris hydrochloride, pH 7.5, at 37°C for 15 min. Then the cells were washed with medium, and RNA (20 to 30 μg) diluted in culture medium was applied to the monolayer (about 0.1 ml per 10^6 cells) and incubated for 1 h at 37°C. Finally, the cells were washed and overlaid with agar, and plaques were visualized as described previously (4). Plaque formation was RNase sensitive and dependent on treatment with DEAE-dextran.

To quantitate the inhibition of FMDV production by ribavirin, monolayers of BHK-21 cells lytically infected with FMDV C_1 or of the persistently infected line C_1-BHK-Rc1 (4) were treated with increasing concentrations of ribavirin in the culture medium. The MIC (ribavirin concentration that reduced the virus yield to 50% measured 24 to 48 h after addition of the drug) was 30 to 50 μg/ml for a lytic infection and 3 to 6 μg/ml for the persistent infection. Concentrations of ribavirin of 150 μg/ml or higher resulted in a decrease in virus production and infectious intracellular RNA, below detectability (Table 1). The RNA transfection assay permits

TABLE 1. Analysis of C_1-BHK-Rc1 cells treated or not treated with ribavirin[a]

Cells	Infectivity	
	Culture medium (PFU/ml)	Intracellular RNA (PFU/mg of RNA)[b]
Treated		
Passage 22	<1	<1
Passage 45	<1	<1
Untreated		
Passage 22	$4.0 (\pm 1.5) \times 10^5$	1,691 ± 109
Passage 45	$3.6 (\pm 1.8) \times 10^4$	193 ± 27

[a] Monolayers (1×10^6 to 1.5×10^6 cells) were treated with ribavirin (150 μg/ml) for 72 h. Then the cells were washed, passaged at least three times, and grown as required for the assays. Parallel cultures were handled identically except that ribavirin was omitted. At the concentrations used, ribavirin was cytostatic but not cytotoxic for BHK-21 cells, as reported previously (8, 11). At least 80% of the cells were viable after ribavirin treatment, as indicated by trypan blue staining. Values are the average of at least three determinations ± SD.

[b] For RNA infectivity assays, 1 mg of RNA from 2×10^8 to 4×10^8 cells was fractionated by sucrose gradient sedimentation. The PFU given are the sum of the number produced by 22 to 24 gradient fractions. Transfections with mixtures of decreasing amounts of virion RNA and constant amounts of cellular RNA indicated that the lower limit of detection was 0.2 to 0.4 PFU/cell. RNA extracted from purified virions yielded 10^5 to 2×10^5 PFU/μg.

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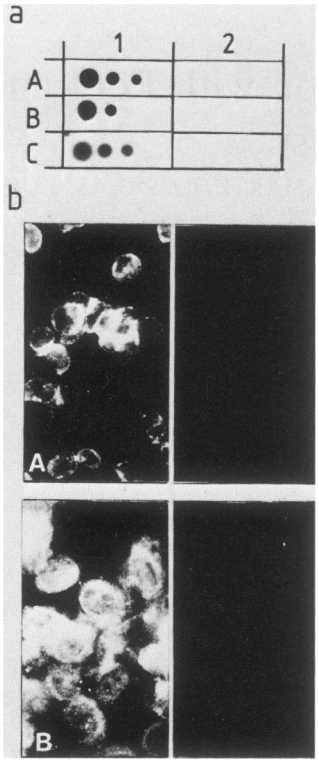

FIG. 1. (a) Dot-blot hybridizations of total cellular RNA to FMDV cDNA from plasmid pBR-VFAC$_1$-18.5, which spans the viral replicase gene at the 3′ end of the genome (7). The indicated amount of RNA was applied to the first dot and 10-fold serial dilutions to successive dots. (Row A) 1, C$_1$-BHK-Rc1 passage 22 RNA (25 μg); 2, RNA from the same cells (50 μg) treated with ribavirin (150 μg/ml) for 72 h. (Row B) 1, C$_1$-BHK-Rc1 passage 52 RNA (30 μg); 2, RNA from the same cells (60 μg) treated with ribavirin. (Row C) 1, RNA from BHK-21 cells infected with FMDV C$_1$ at a multiplicity of infection of 5 to 10 PFU/cell and extracted at 4 h postinfection (10 μg); 2, RNA from BHK-21 cells (60 μg). (b) Indirect immunofluorescence, assayed with anti-FMDV rabbit serum as described previously (4). Left, cells not treated with ribavirin; right, cells treated with ribavirin. A, C$_1$-BHK-Rc1 passage 22; B, C$_1$-BHK-Rc1 passage 52.

finding an average of one infectious RNA molecule per cell. In ribavirin-treated cultures no FMDV genomic sequences or antigens could be revealed by dot-blot hybridization to a cDNA probe or by indirect immunofluorescence, respectively (Fig. 1). After removal of the drug, no renewed FMDV production occurred during at least 20 serial passages, as expected from the elimination of infectious viral genomes. We conclude that treatment with ribavirin cured the persistently infected C$_1$-BHK-Rc1 cells of infectious FMDV RNA. Several ribavirin-cured cell lines have been derived. They have been passaged, frozen, thawed, and regrown by the same procedures used for BHK-21 cells without detectable loss of viability. The response of cured cells to infection by FMDV, now under investigation, is extremely complex and suggests a rapid phenotypic evolution of the virus during persistence (4–6) and what appears to be coevolution of the host cells (unpublished results).

Although the antiviral activity of ribavirin has been well documented (3, 10, 11), the results reported here are, to our knowledge, the first instance of curing by ribavirin of a cell line persistently infected with an animal virus. It is noteworthy that BHK-21 cells persistently infected with mumps virus could not be cured with doses of ribavirin threefold higher (8) than those effective on C$_1$-BHK-Rc1 cells. In fact, in infections with mumps virus, ribavirin was more effective in acute than in persistent infections (8), a result opposite to our findings with FMDV. Interpretation of such differences must await clarification of the mechanism of action of ribavirin, thought to involve inhibition of the biosynthesis of guanylic acid nucleotides (3, 11). For FMDV, the early results of Bachrach and Werner showed some inhibition by ribavirin of lytic infections of FMDV in cell culture and of mice in vivo (quoted in Tables 4 and 6 of reference 10). We have shown that administration of ribavirin to mice delays death and increases the number of survivors of infection with FMDV C$_1$ (unpublished experiments). Thus, research on the action of ribavirin on carrier cattle is encouraged by (i) the 10-fold-greater sensitivity of persistently infected than lytically infected cells to the drug; (ii) several common features of the in vitro system with the in vivo FMDV carrier state (selection of virus variants [miniplaque, temperature sensitive, etc.], temporary, not permanent, FMDV production, maintenance of persistence in the presence of anti-FMDV antibodies [4, 13]), and (iii) carrier cattle constitute a major reservoir of FMDV, probably uncontrollable by vaccination alone, and for which no prophylactic therapeutic treatments have been suggested.

We thank M. Dávila for technical assistance and J. R. Allen for comments. We are indebted to Viratek for supplying us with ribavirin and to Laboratories Sobrino S.A. for help with in vivo experiments.

Research supported by Comisión Asesora para el Desarrollo de la Investigación Científica, Fondo de Investigaciones Sanitarias, Consejo Superior de Investigaciones Científicas, and Caja de Ahorros y Monte de Piedad de Madrid, Spain.

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