Inhibition of a Vesicular Stomatitis Virus Mutant by Rifampin

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Rifampin reversibly inhibits the intracellular replication of a VSV mutant, whereas a revertant variant selected from this viral population, as well as the wild strain, are not affected by this drug. Rifampin inhibits transcriptase activity of the sensitive mutant only and, consequently, total viral RNA synthesis decreases significantly in the cells.

A rifamycin derivative, rifampin, has been found to inhibit bacterial RNA synthesis (3, 12). This antibiotic blocks RNA chain initiation after binding of the bacterial RNA polymerase to DNA.

Rifampin also inhibits the growth of vaccinia virus at a late stage of its replicative cycle (4, 11). The sensitive step is not yet completely identified (6, 10); however, the mechanisms with which rifampin blocks viral and bacterial multiplication may not be identical.

We have previously shown that rifampin reversibly inhibits the intracellular replication of a mutant vesicular stomatitis virus (VSV) strain (9). In this report, we will study the mechanisms of this inhibition.

MATERIALS AND METHODS

Cell cultures. Mouse L cells, African green monkey cells (line BSC) and chicken embryo fibroblasts (CEF) were grown in Eagle medium (MEM) 0111 supplemented with tryptose phosphate broth (2.95 g/liter) and 10% heat-inactivated calf serum.

Viruses. The Indiana strain of VSV was grown in L cells. A large plaque mutant of VSV obtained in this laboratory was employed. Cells were infected with 10^{-1} PFU per cell in Eagle medium without calf serum. The virus produced was harvested after 24 h of incubation at 37°C. Infectivity assays were performed by routine plaque titration.

Rifampin-resistant mutants were selected by 4 to 6 successive plaque purifications of virus grown in the presence of rifampin at a concentration of 100 µg of medium per ml.

VSV virions were concentrated by precipitation with polyethylene glycol 6000 and purified using a DEAE-cellulose column chromatography, as described by McSharry and Wagner (8).

Antibiotics. Rifampin was supplied by Lancini Lepetit Spa Milan (Italy) through the courtesy of S. Fürész. Prior to use, the drug was dissolved in phosphate broth (PBS) buffer and diluted in MEM medium to a suitable concentration.

Actinomycin D (a gift of Merck Sharp and Dohme Research Laboratories, Rahway, N. J.) was dissolved in a 5% ethanol PBS buffer at a concentration of 1 mg/ml and diluted with medium.

Determination of total viral RNA synthesis. A monolayer of L cells, grown in plastic petri dishes (35 by 10 mm), was infected with VSV at a multiplicity of 10 PFU/cell. After an adsorption period of 1 h at 37°C, rifampin (200 µg/ml), actinomycin D (2 µg/ml), and [5-³H]uridine (21 Ci/mM; 2 µCi/ml) were added to the infected cells.

The cells were harvested at hourly intervals. Cumulative incorporation of radioactive uridine into trichloroacetic acid-precipitable fraction was assayed in a Packard Tri-Carb liquid scintillation counter.

[5-³H]uridine (21 Ci/mM) was purchased at the Centre de l'Energie Atomique (C.E.A.) Saclay (France).

VSV-transcriptase assay. The enzyme was assayed according to the technique described by Baltimore, Huang, and Stampfer (1). In our assays the tests were performed at 32°C and pH 7.2. Amounts of viral protein ranging from 10 to 40 µg were determined by the method of Lowry (7) and added to the reaction mixture.

As a tracer, [³H]GTP (0.25 mCi/0.027 mg), purchased at C.E.A. Saclay (France), was employed.

RESULTS

The VSV strain sensitive to rifampin was a large plaque mutant of the Indiana strain. However, during the passages, small or large plaque mutants were obtained. There was no correlation between the size of the plaque and the sensitivity of the virus to the antibiotic.

The inhibitory effect of rifampin on VSV replication was independent of the cell system used. In parallel sets of preparations, this antibiotic at the same concentration did not affect the replication of encephalomyocarditis virus (EMC) in L cells or of poliovirus in BSC cells (Table 1).

The rate of inhibition was proportional to the

concentration of rifampin used (Fig. 1). In uninfected cells, for concentrations of the drug lower than 200 $\mu\text{g/ml}$ after 24-h treatment, no change in the intracellular RNA or protein pool was observed by cumulative incorporation of [^3H]uridine and [^{14}C]amino acid in the trichloroacetic acid-precipitable fraction. For 200 μg of rifampin per ml, RNA synthesis was inhibited by 12% and protein synthesis by 3%. For rifampin concentrations higher than 200 $\mu\text{g/ml}$, after a 10-h incubation, a slight morphological change appeared in the cells. Intracellular RNA and protein synthesis were inhibited 20 to 30% after 18 h with rifampin (300 $\mu\text{g/ml}$).

Reversibility of the antiviral action of rifampin. After adsorption of VSV on L cells, one set of cell preparation was treated with rifampin for 18 h. Another set was treated with rifampin for 18 h and then with MEM for 7 h. The viral yield obtained for the two sets was compared to suitable controls. A significant but incomplete reversal of the antiviral effect of rifampin was observed after withdrawal of the drug (Table 2).

Effect of rifampin during the first replicative cycle. VSV sensitive to rifampin (VSV-RIF⁺) or resistant to it (VSV-RIF⁻) were measured by yield inhibition in the presence of the drug. Viral yields were titrated every hour during the first replicative cycle (10 h). A significant difference between treated and untreated virus appeared with VSV-RIF⁺: 5 to 7 h after infection the inhibition was about 30-fold (Fig. 2A). The multiplication of VSV-RIF⁻ was unaffected by rifampin (Fig. 2B).

In further experiments we investigated the mechanisms of the antiviral effect of rifampin.

Viral RNA synthesis in the presence of rifampin. In uninfected cells treated with rifampin, no significant change in the cumulative incorporation of [^3H]uridine was observed.

L cells were infected with VSV and rifampin added 1 h later. The viral RNA synthesis (determined as shown in Materials and Methods) was inhibited by about 50% from 4 to

FIG. 1. Inhibition of VSV replication by different concentrations of rifampin. Rifampin was added to L cells after viral adsorption. EMC virus was used as a control of cytotoxicity. Cells were infected with VSV and EMC at an MOI of 1 PFU/cell. The viral yields were titrated after 1 replicative cycle.

7 h after infection. A possible decrease of [^3H]uridine uptake in the presence of the drug was not responsible for these results, as shown in uninfected controls (Fig. 3).

Sequential addition of rifampin. Rifampin was added at hourly intervals during the first VSV replicative cycle. Viral yields were titrated 11 h after the infection of cells. The inhibition of VSV multiplication was maximal when the drug was added 1 h after infection and decreased rapidly (Fig. 4). A possible effect of rifampin on VSV transcription was, therefore, studied in the following experiments.

TABLE 1. Inhibition of VSV replication by rifampin in different cell systems^a

Cell system	Virus titer (PFU/ml)					
	VSV		Poliovirus		EMC	
	Control	RIF	Control	RIF	Control	RIF
L	3.2×10^7	2.5×10^4	ND	ND	10^8	2.6×10^8
BSC	2×10^6	7.7×10^2	9.5×10^5	7.4×10^5	ND	ND
CEF	3.7×10^6	1.3×10^8	ND	ND	1.8×10^6	10^6

^a Rifampin (RIF), 100 $\mu\text{g/ml}$, was added to the culture medium 1 h after viral adsorption. Cells were infected with VSV, EMC, and poliovirus at an MOI of 1 PFU/cell. The viral yield was titrated after 1 replicative cycle. First passage CEF cells were used in these experiments. ND, Not done.

TABLE 2. *Reversal of the antiviral action of rifampin*^a

Virus	Virus titer (PFU/ml)				
	Medium 18 h	Rifampin 18 h	Medium 18 h and medium 7 h	Rifampin 18 h and medium 7 h	Rifampin 18 h and rifampin 7 h
VSV	3.2×10^7	1.7×10^4	1.3×10^7	3.5×10^6	2.5×10^4
EMC	3.7×10^8	2.1×10^8	2.5×10^8	2.1×10^8	2×10^8

^a Rifampin was added 1 h after viral adsorption at a concentration of 100 $\mu\text{g/ml}$. When rifampin was removed, L cells were washed with MEM and incubated for 7 h with medium or rifampin (100 $\mu\text{g/ml}$). VSV and EMC were used at an MOI of 1 PFU/cell.

FIG. 2. Effect of rifampin during the first replicative cycle of VSV. L cells were infected with VSV at an MOI of 1 PFU/cell. After 1 h of viral adsorption, rifampin (100 $\mu\text{g/ml}$) was added for 10 h. Viral yield was assayed for infectivity at hourly intervals. (A) VSV-RIF⁺: treated (●) and untreated (■) with rifampin. (B) VSV-RIF⁻: treated (●) and untreated (■) with rifampin.

Inhibition of VSV transcriptase activity by rifampin. Transcriptase activity was tested *in vitro* with purified virus. In the first experiment, rifampin was added to the reaction mixture at the beginning of incubation (Table 3). As previously observed by Baltimore et al. (1), rifampin did not inhibit the incorporation of [³H]GMP either in the wild or in the RIF⁻ strain of VSV. The transcriptase activity of the

FIG. 3. Cumulative viral RNA synthesis in the presence and absence of rifampin. 2.10^6 L cells uninfected (Δ) or infected ($\circ$) with VSV-RIF⁺ were treated 8 h with rifampin (200 $\mu\text{g/ml}$). At 1 h postinfection, [³H]uridine was added at a concentration of 2 $\mu\text{Ci/ml}$. Intracytoplasmic RNA of control cells uninfected ($\blacktriangle$) or infected ($\bullet$) with VSV was similarly labeled without rifampin.

RIF⁺ mutant or of a subclone selected from this population was greatly inhibited.

In the second experiment, the kinetics of incorporation of [³H]GMP was examined when rifampin (4 μg) was added at different time intervals to the reaction mixture. VSV-RIF⁺ was incubated with the components of the reaction assay at 32 C for 100 min (Fig. 5). The transcriptase activity was inhibited when rifampin was added 10 and 30 min after the beginning of the experiments. When added at 40 and 60 min, rifampin seemed to stimulate slightly the incorporation of [³H]GMP.

FIG. 4. Sequential addition of rifampin. L cells were infected with VSV-RIF⁺ (MOI = 1 PFU/cell). Rifampin (100 µg/ml) was added at hourly intervals. Plaque titrations were performed after 11 h of infection.

TABLE 3. Effect of rifampin on the rate of [³H]GMP incorporation in vitro

Assays ^a	Rifampin final concn		
	0	2 µg	4 µg
VSV-RIF ⁺	51.95 ^b	35.33	21.30
Subclone VSV-RIF ⁺	45.38	6.35	0
Wild VSV	36.0	34.37	36.0
VSV-RIF ⁻	43.15	41.84	43.03

^a Assays were incubated for 100 min at 32 C. Controls were tested in parallel by using the solvent of rifampin. In the absence of virus, [³H]GMP incorporation was the same (less than 4%) in unincubated samples with 0, 2, or 4 µg of rifampin.

^b Picomoles of [³H]GMP incorporation per milligram of viral protein per hour.

Inhibition of transcriptase activity by rifampin acts probably at an early stage of transcription. However, when added after a 40- or 60-min incubation period, [³H]GMP might incorporate only in RNA strands initiated before the addition of rifampin.

FIG. 5. Kinetics of incorporation of [³H]GMP when rifampin was added to the reaction mixture at 0, 10, 30, 40, and 60 min. Total enzyme assay was 300 µliters, and 25-µl fractions were assayed for radioactivity. The value of an unincubated sample (12 counts/min) was subtracted from all of the experimental values. Symbols: ●, control without rifampin; *, rifampin added at 0 min; ○, rifampin added at 10 min; ■, rifampin added at 30 min; □, rifampin added at 40 min; ▲, rifampin added at 60 min.

DISCUSSION

Rifampin reversibly inhibits the intracellular replication of a VSV mutant. The experimental data presented here show that the drug decreases the rate of total viral RNA synthesis in the cells and inhibits viral transcriptase activity when assayed in a cell-free system.

Some rifampin derivatives known to inhibit reverse transcriptase activity of oncornaviruses similarly inhibit VSV transcriptase (5). For VSV-RIF⁺, the mutation might be situated at the transcriptase level and could perhaps explain the affinity of the enzyme for rifampin.

In summary, our studies seem to indicate that in the case of the mutant VSV strain, the primary change which causes rifampin sensitivity might be located at the transcription level. This observation does not exclude, however, the possibility that this drug might have a different effect on other viruses. Events in the maturation of vaccinia virus (6, 10), as well as poliovirus in suspended cell cultures (2), could also

be involved. In the case of VSV, this problem will be further investigated using different VSV strains grown in other cell systems.

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