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Ribonucleic Acid Polymerase Induced by Vaccinia Virus: Lack of Inhibition by Rifampicin and α-Amanitin

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The RNA polymerase activity present in the cytoplasm of BHK cells infected with vaccinia virus is not affected by rifampicin or by α-amanitin.

The rifamycins, a group of broad-spectrum antibiotics, have been shown to block bacterial ribonucleic acid (RNA) synthesis (7, 25) by inhibiting the deoxyribonucleic acid (DNA)-dependent RNA polymerase (nucleosidetriphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) (20, 27), with which they form a very stable complex in a well-defined stoichiometric ratio (26, 27).

The inhibition of the bacterial RNA polymerase seems to be responsible for the antibacterial activity of this group of antibiotics (12).

In addition it has been demonstrated that the rifamycins do not affect the RNA polymerase of mammalian origin (25, 28).

Recently Heller et al. (8) and Subak-Sharpe, Timbury, and Williams (24) have discovered that rifampicin, a semisynthetic member of the rifamycin family (14), selectively inhibits the replication of some poxviruses (vaccinia virus and cowpoxvirus) in tissue cultures.

As poxviruses possess a DNA-dependent RNA polymerase as an integral component of the virion (11, 17), it was logical to assume that the antipoxvirus activity of rifampicin could be the consequence of the inhibition of the virus-specific transcriptase (8, 24).

α-Amanitin, the most powerful toxin of the toadstool *Amanita phalloides* (30), has been shown to impair mammalian RNA synthesis in vivo and to depress, both in vivo and in vitro, the activity of mammalian DNA-dependent RNA polymerase activated by Mn^{2+} and ammonium sulfate (22). α-Amanitin is unable to prevent bacterial growth and the replication of various RNA and DNA viruses, including the vaccinia virus (4).

The results reported in this paper demonstrate that rifampicin and α-amanitin do not affect the activity of the DNA-dependent RNA polymerase induced in the cytoplasm of cultured cells infected by vaccinia virus.

BHK 21/C 13 (baby hamster kidney) (23) cell cultures were grown in stationary Roux-type bottles in Eagle's basal medium (3) supplemented with 10% calf serum. The cells were transferred to serum-free Eagle's basal medium and were infected with vaccinia virus strain IHD (ATCC VR156) at a multiplicity of infection of one plaque-forming unit per cell. Addition of rifampicin (100 μg/ml) to the cell culture medium completely prevented the cytopathic effect and reduced by 99% the yield of cell-associated virus (*see* below), as estimated by the plaque-formation method. After 20 hr of infection the medium was removed, the cell monolayer was washed twice with 0.85% NaCl, and the cells were scraped off the bottles with a rubber policeman and washed twice with the same solution. After swelling for 10 min in 0.01 M tris-(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0, the cells (approximately 2.5×10^7/ml.) were disrupted with a Dounce homogenizer and a "nuclear" and "cytoplasmic" fraction were obtained (19). Nuclei were resuspended to the initial volume in Tris buffer; Triton X-100 was added to the cytoplasmic fraction at final concentration of 1%.

RNA polymerase activity of the nuclear fraction was assayed in a low ionic strength system (29) containing Mg^{2+} (referred to as 5 mM Mg^{2+} system) and in a high ionic strength system (18, 29) containing Mn^{2+} and ammonium sulfate [referred to as $Mn^{2+}/(NH_4)_2SO_4$ system]. The enzyme activity of the cytoplasmic fraction was assayed in the system described by Pitkanen et al. (19) (referred to as 12 mM Mg^{2+} system).

DNA was determined by the method of Bur-

ton (2) and protein by the method of Gornall, Bardawill, and David (6) or by that of Lowry et al. (13).

The RNA polymerase activity of the nuclear fraction was two to three times higher in infected than in uninfected cells, when assayed at low ionic strength (Table 1). This difference was not observed when the enzyme was assayed at high ionic strength, thus indicating that the higher polymerase activity of infected nuclei in the 5 mM Mg^{2+} system was due to contamination by cytoplasmic residues containing virus-induced enzyme. [The virus-induced polymerase is not stimulated by high ionic strength (*see* below)].

The activity of nuclear RNA polymerase in the $Mn^{2+}/(NH_4)_2SO_4$ system was greatly impaired by addition of 0.025 μg/ml of α-amanitin, whereas rifampicin (50 μg/ml) did not significantly affect nuclear RNA polymerase in both the reaction systems employed.

A very high RNA polymerase activity was detected in the cytoplasmic fraction of cells infected with vaccinia virus: the small activity detectable in the cytoplasmic fraction of uninfected cells was considered the result of contamination by nuclear residues.

In the 12 mM Mg^{2+} system the activity of the virus-induced enzyme was completely unaffected by rifampicin up to 300 μg/ml, and by α-amanitin up to 0.050 μg/ml, i.e. at a concentration three times higher than that causing maximal inhibition in mouse liver nuclei (22). The virus-induced

TABLE 1. *Effect of rifampicin and α-amanitin on the ribonucleic acid (RNA) polymerase activity normally present in uninfected cells (nuclear fraction) and induced by vaccinia virus infection (cytoplasmic fraction)*

Source of enzyme	Addition	RNA polymerase activity[a]		
		5 mM Mg^{2+} system[b]	$Mn^{2+}/(NH_4)_2SO_4$ system[b]	12 mM Mg^{2+} system[c]
Nuclei from normal cells	—[d]	6.8	31.2	
	Rifampicin, 50 μg/ml	4.9	24.1	
	α-Amanitin, 0.025 μg/ml[e]	3.5	7.8	
Nuclei from infected cells	—	15.5	22.7	
	Rifampicin, 50 μg/ml	15.5	20.4	
	α-Amanitin, 0.025 μg/ml	14.7	7.8	
Cytoplasm from normal cells	—			672
Cytoplasm from infected cells	—			13,350
	Rifampicin, 100 μg/ml			15,146
	α-Amanitin, 0.050 μg/ml			16,175
	0.28 M Ammonium sulfate			303
Cytoplasm from infected cells (separate experiment)	—			4,022
	Rifampicin, 300 μg/ml			4,165

[a] RNA polymerase activity is expressed as counts per minute per microgram of DNA per 10 min for the 5 mM Mg^{2+} system and the $Mn^{2+}(NH_4)_2SO_4$ system, and counts per minute per milligram of protein per 40 min for the 12 mM Mg^{2+} system.

[b] The 5 mM Mg^{2+} system contained in a final volume of 0.5 ml: 50 μmoles of Tris-hydrochloride buffer (*p*H 8.5), 2.5 μmoles of $MgCl_2$, 10 μmoles of cysteine, 3 μmoles of NaF, 0.3 μmole each of guanosine triphosphate (GTP), cytosine triphosphate (CTP), and uridine triphosphate (UTP), 0.02 μmole of adenine triphosphate (ATP) containing 0.1 μc of [8-^{14}C]ATP, and 0.1 ml of nuclear suspension. The $Mn^{2+}/(NH_4)_2SO_4$ system contained, in a final volume of 0.5 ml: 50 μmoles of Tris-hydrochloride buffer (*p*H 7.5), 2 μmoles of $MnCl_2$, 0.035 ml of ammonium sulfate saturated at room temperature, and brought to *p*H 7.5 with diluted ammonia solution (final concentration in the assay mixture 0.28 M), 0.9 μmole each of GTP, CTP, and UTP, 0.06 μmole of ATP containing 0.3 μc of [8-^{14}C]ATP, and 0.1 ml of nuclear suspension. With both systems incubation was at 37 C for 10 min.

[c] The 12 mM Mg^{2+} system contained in a final volume of 0.25 ml: 30 μmoles of Tris-hydrochloride buffer (*p*H 8.5), 3 μmoles of $MgCl_2$, 2 μmoles of mercaptoethanol, 100 μg of DNA (calf thymus), 1 μmole each of GTP, CTP, and UTP, 0.105 μmoles of ATP containing 0.1 μc of [8-^{14}C)ATP, and 0.1 ml of cytoplasmic fraction (about 3.8 mg of protein per ml). The mixture was incubated at 37 C for 20 or 40 min.

[d] No addition.

[e] Rifampicin or α-amanitin, when present, was added to mixtures containing all components except nucleotides, which were added after 2 min of preincubation at 25 C.

polymerase was completely inhibited upon addition of ammonium sulfate at the concentration (0.28 M) giving optimal stimulation of the nuclear enzyme (18). The peculiar sensitivity of the virus-induced enzyme to ammonium sulfate prompted a second series of experiments in which the effect of various concentrations of ammonium sulfate (from 6.5×10^{-2} to 10^{-7}) added to the 12 mM Mg^{2+} system was studied. An activation of the virus-induced polymerase was observed only in the presence of ammonium sulfate at the final concentrations from 10^{-2} to 10^{-7} in one experiment and from 10^{-4} to 5×10^{-5} in a second experiment.

When the action of rifampicin (300 μg/ml) and of α-amanitin (0.05 μg/ml) in the presence of ammonium sulfate at concentrations giving optimal stimulation was investigated, again no impairment of the virus-induced RNA polymerase was observed.

We found, in agreement with the findings of Kates, Dahl, and Mielke (11) and of Pitkanen et al. (19), that the virus-induced RNA polymerase was active in vitro only after detergent treatment (*see* above) and, therefore, represents the enzyme which, in all probability, is packaged into the mature virion (10). The data obtained in our experiments demonstrate that the virus-induced RNA polymerase activity does not show any sensitivity to rifampicin when tested under various experimental conditions. Therefore, it seems possible to conclude that the mechanism underlying the antipoxvirus activity of rifampicin does not involve the impairment of the virus-induced RNA polymerase. [After this paper had been submitted for publication, similar conclusions were reached by Moss, Katz, and Rosenblum (16) and by Ben-Ishai et al. (1)] Moreover, the fact that α-amanitin did not affect the activity of the RNA polymerase induced by the vaccinia virus represents a distinctive character which differentiates the virus-induced enzyme from the RNA polymerase or at least one of the RNA polymerase activities (15) present in the mammalian nucleus.

Another result which further substantiates the peculiarity of the RNA polymerase induced in the cell infected by vaccinia virus is represented by the salt concentrations stimulating the enzyme activity, which were much lower than those stimulating mammalian (9, 18) or bacterial (5, 21) RNA polymerases.

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