Effect of Rifampicin on Poxvirus Protein Synthesis

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Poxvirus strains differ with respect to the effect of rifampicin on viral protein synthesis. Rifampicin severely depressed vaccinia-directed protein synthesis but had little effect on the rate of cowpox-directed protein synthesis, including one late virus-induced enzyme. The spectrum of polypeptides synthesized in cowpox-infected cells was similar in the presence or absence of rifampicin except for one significant difference. After removal of rifampicin, virusslike particles assembled to some extent, even when protein synthesis was inhibited by cycloheximide. However, reversal was more extensive if protein synthesis was allowed.

Inhibition of vaccinia replication by rifampicin has been established by several groups (2, 14). Accumulated evidence for the mechanism of rifampicin inhibition indicates that early viral functions are not impaired (3), viral deoxyribonucleic acid (DNA) synthesis is not greatly affected (3, 8), and late viral messenger ribonucleic acid (mRNA) synthesis is normal with respect to rate of synthesis (3, 8), median sedimentation coefficient (1, 8), and association with polysomes (1). Some differences of opinion exist concerning the effect of rifampicin on synthesis of late viral proteins. Becker's group (1) found that rifampicin inhibited incorporation of labeled amino acids into polysomes of vaccinia-infected cells, and concluded that rifampicin selectively inhibited late viral protein synthesis. Moss' group (8) found that all structural vaccinia proteins are made in the presence of rifampicin. They presented evidence that rifampicin blocks assembly of viral components and suggested that assembly failure might depress the rate of viral protein synthesis (9).

This report concerns the different responses of two poxvirus strains to rifampicin and is relevant to an understanding of the mechanism of the action of the antiviral agent.

MATERIALS AND METHODS

Cells and virus. HeLa-S3 cells were maintained in suspension in Eagle's medium supplemented with 5% fetal calf serum. The Brighton strain of cowpox was propagated in embryonated hen eggs. Vaccinia WR was propagated in HeLa-S3 cultures. Techniques for infection of cells have been described (5). Unless otherwise stated, the input multiplicity of infection was 10 plaque- or plaque-forming units (PFU) of either cowpox or vaccinia WR per cell. Both virus preparations were partially purified by modifications of the procedures of Planterose et al. (11). Titration of stock virus was approximately 5 × 10^6 PFU for cowpox and WR.

Rate of protein synthesis. Infected cells were diluted, after adsorption of virus, in Eagle's medium which contained one-sixth of the normal amino acid concentration and was supplemented with 10% serum. Portions containing 5 × 10^6 cells were incubated with L-leucine-4,5-^3H (specific activity, 40 Ci/mmol; 2 μCi/ml of culture) for 20 min. Cells were harvested, washed once in cold tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.10 M, pH 7.8), and then disrupted in reticulocyte-swelling buffer (10) by Dounce homogenization. Nuclei were removed by centrifugation (500 × g, 3 min), the cytoplasmic fractions were acidified (5% trichloroacetic acid), and the precipitate was collected on glass-fiber filters for assay by scintillation spectrometry.

Assay of RNA polymerase and deoxyribonuclease. The cytoplasmic fraction obtained by disruption of infected cells was used to assay RNA polymerase, as recently described in detail (3). Activity is recorded as counts per minute of uridine-5-monophosphate incorporated per milligram of protein per hour. Acid deoxyribonuclease activity of the extracts was measured by the method of McAuslan and Kates (6). Briefly, the crude extract was adjusted to 0.1% sodium deoxycholate. A small sample (20 μl) was mixed with 10 μl of 70% ammonium sulfate, 0.35 ml of acetate buffer (pH 4.8), and 10 μg of heat-denatured 3H-DNA from Escherichia coli (10 μg, 30,000 counts/min). The mixture was incubated (30 min, 37°C), and the acid-soluble counts were determined by liquid scintillation spectrometry. Activity is expressed as counts per minute rendered acid-soluble per 20 μg of protein per 30 min.

Gradient centrifugation of viral particles. Infected cells were disrupted in hypotonic medium KBM [10^-2 M Tris-hydrochloride (pH 7.4), 10^-2 M KCl, 10^-2 M ethylenediaminetetraacetate (EDTA)]. After removal of nuclei by centrifugation, the extracts were usually treated with deoxyribonuclease (see Results)
and then layered on gradients of 50 to 70% (w/v) sucrose in Tris-hydrochloride (10^{-2} \text{ M}, \text{pH} 7.4, plus 0.005 \text{ M EDTA}). After centrifugation (95,000 \times g, 120 min), the gradients were fractionated, and acid-precipitable counts in each fraction were determined.

**Gel electrophoresis of polypeptides.** Polypeptides were analyzed by modifications of methods presented by Maizel (7). Viral particles were collected from sucrose gradients. Virus was pelleted by centrifugation (95,000 \times g, 30 min), and the pellet was dispersed in 0.3 ml of Tris-hydrochloride (0.1 \text{ M}, \text{pH} 8.0) which was adjusted to the following concentrations: sodium dodecyl sulfate, 2.5%; urea, 1.0 \text{ M}; EDTA, 3 \text{ mm}; and dithiothreitol, 0.17 \text{ M}. The mixture was incubated (37 \degree C, 45 min; then 100 \degree C, 100 sec), and then was dialyzed overnight against phosphate buffer (0.005 \text{ M}, \text{pH} 7.2, with 0.5 \text{ M urea and 0.1\% sodium dodecyl sulfate}). Prior to electrophoresis, samples were made to 5\% sucrose, heated (60 \degree C, 30 min), cooled, and applied to gels. Cytoplasmic extracts of cells were processed similarly.

Samples were subjected to electrophoresis on 8.5\% acrylamide gels (7 by 160 mm, 5 \text{ ma per gel, 20 hr}), after which the gels were sliced (1-mm slices). Gel slices were incubated in a Nuclear-Chicago Solubilizer (0.5 ml, 16 hr, 65 \degree C). Liquiflor scintillation fluid (7 ml) was added, and radioactivity was determined after gel slices had been allowed to swell in the scintillation fluid (4 hr).

**Rifampicin.** Rifampicin was obtained from Ciba Pharmaceutical Co. It was first dissolved in a few drops of dimethyl sulfoxide and then diluted 10-fold in 0.01 \text{ M ascorbic acid}. When used, the inhibitor was added to cultures 30 min after adsorption of virus.

**RESULTS**

**Inhibition of cowpox replication.** Evidence for inhibition of vaccinia replication by rifampicin has been presented (8, 14). We have confirmed this, and we found that replication of cowpox in HeLa-S3 cells is also inhibited by rifampicin (Fig. 1). Under single-cycle growth conditions, the yield of infective cowpox 20 hr postinfection was approximately one-third the usual yield of vaccinia virus. This probably reflects a higher ratio of elementary bodies to infective particles for cowpox or inefficient maturation, rather than a difference in rate of synthesis of major viral components.

**Rate of viral protein synthesis.** Measurement of the rate of incorporation of tritiated leucine into acid-precipitable form in the cytoplasmic fraction of infected cells gives a measure of the rate of viral protein synthesis, because host protein synthesis and RNA synthesis are rapidly arrested after poxvirus infection (13). In this way, we have determined the effect of rifampicin on the rate of vaccinia (WR) and cowpox protein synthesis in infected HeLa cells (Fig. 2). Rifampicin depressed markedly the rate of protein synthesis in WR-infected cells. A corresponding depression was not produced in cowpox-infected cells; leucine must have been incorporated into cowpox-directed proteins rather than cell proteins, because ultraviolet-inactivated virus rapidly depressed incorporation of leucine (Fig. 2a). It seems clear that these experiments, medium with only one-sixth the concentration of amino acids present in regular growth medium was used to increase the proportion of isotopic leucine incorporated. Essentially the same kinetics of incorporation were found when complete medium was used, although the specific activity was lower and, as expected, the rate of incorporation of leucine into uninfected cells remained constant during the course of the experiment.

**Induction of late enzyme activities.** At least two late enzyme activities, DNA-dependent RNA polymerase and deoxyribonuclease, are induced by poxvirus infection (4). There is evidence that these activities are structural components of the viral particles (12).

Induction of the RNA polymerase activity was inhibited by rifampicin in both WR and cowpox systems (3). McAuslan proposed that rifampicin binds with a soluble form of the polymerase (3), and that this prevents active combination with poxvirus DNA. If, as it appears, the RNA polymerase is active only as part of a viral structure, then rifampicin inhibition of induction could be explained by Moss's data (9) as failure in assembly of the minimal subviral structure necessary for activity. Other data
suggest that there is no polymerase activity because there is negligible late protein synthesis (1). Although rifampicin had negligible effect on the rate of protein synthesis in cowpox-infected cells, it was of interest to assay a late enzyme activity as an indicator of the quality of late protein synthesized. The effect of rifampicin on induction of the two late cowpox-induced enzyme activities in question was determined. Cells were infected with cowpox (10 PFU/cell). To half of the infected culture, rifampicin (100 μg/ml) was added 30 min postinfection. Enzyme activities of the cytoplasmic fraction of such cultures were determined after disruption of the cells (see Materials and Methods). We found that cowpox-induced RNA polymerase activity was not detectable in extracts of rifampicin-treated cells, whereas the induction of acid deoxyribonuclease proceeded at almost the normal rate (Fig. 3). Furthermore, it is of interest that the nuclease activity, recently reported to be incorporated into virions, was found in the particulate fraction (100,000 × g precipitate), even in extracts of rifampicin-treated cells. In contrast, we could not detect induced deoxyribonuclease in rifampicin-treated WR-infected cells.

**Assembly of particles in rifampicin-inhibited systems; production of “rifampicin-particles.”** Cells were infected with cowpox or WR at an input of 10 PFU per cell. Rifampicin (100 μg/ml) was added after adsorption. Tritiated thymidine (specific activity, 6.7 Ci/m mole; 0.5 μCi/ml of culture) was added to each culture. At 20 hr postinfection, the cytoplasmic fraction from cells was prepared by disruption of cells in hypotonic medium KBM. The extract was adjusted to 5 × 10⁻³ M MgCl₂, pancreatic deoxyribonuclease was added (100 μg/ml), and the extract was incubated (60 min, 37°C). The Mg²⁺ concentration was
reduced to zero by chelation with EDTA; the extract was subjected to brief sonic vibration (1 min, full power, MSE 60-cycle sonic disintegrator) and centrifuged on sucrose gradients as described in Materials and Methods. Under such conditions, infective particles labeled with tritiated thymidine banded at the positions indicated (Fig. 4), and about 50% of the DNA (viral) in the cytoplasmic fraction was deoxyribonuclease-resistant. In contrast, 90 to 95% of the DNA of the cytoplasmic fractions of rifampicin-treated cells was degradable by deoxyribonuclease, and the yield of DNA-containing particles was greatly reduced. However, in both the WR and cowpox systems, we found routinely a small yield (about 5%) of DNA-containing particles even after rifampicin treatment. We shall refer to these as “rifampicin particles.” They banded at a position in the gradient corresponding to a slightly greater density than normal viral particles and appeared to be more permeable to phosphotungstic acid than were normal viral particles (Fig. 5). Upon reversal of the rifampicin inhibition in the presence of cycloheximide, as described by Moss et al. (9), we detected some assembly of particles from components that were synthesized during the rifampicin block (Fig. 6). Reversal of rifampicin without inhibiting protein synthesis allowed efficient assembly of particles that banded at the same position in the gradient as normal particles.

Polypeptides of rifampicin particles. As part of a study on rifampicin particles, they were labeled with a mixture of tritiated amino acids and isolated from sucrose gradients; their polypeptides were then analyzed by gel electrophoresis. The polypeptide profiles thus obtained were compared with those of normal virus particles (Fig. 7). Comparison of cowpox polypeptides with those of the corresponding rifampicin particles revealed one distinct difference. Around the position of peak A of the normal polypeptide profile, two peaks (α1, α2) were invariably found. These two peaks remained associated with the particles after they were recentrifuged through sucrose gradients. The result was confirmed in a number of separate experiments. Some alterations in the relative proportion of other peaks were usually found, but we are not confident that such differences are significant. The apparent loss of peak B from the rifampicin particle profile shown may be due to failure in resolution in the experiment described. Low concentrations of rifampicin (10 μg/ml) did not inhibit cowpox replication and did not alter the polypeptide profile.

Since the rifampicin particles represented at most 10% of the normal particle yield, it could be argued that any abnormality in their polypeptides
Fig. 5. Electron micrographs of particles. (a) Particles from fraction 14 of the gradient described by Fig. 4a. (b) Particles from fraction 11 of the gradient described by Fig. 4a. × 70,000.

does not reflect the polypeptide composition of the cytoplasmic fraction of infected cells. The whole cytoplasmic fraction of rifampicin-treated cowpox-infected cells was compared with that of uninhibited controls by gel electrophoresis (Fig. 8). Again, the two peaks a1 and a5 were found in the whole cytoplasmic extract and in roughly the same proposition, relative to other peaks, as found in isolated rifampicin particles.

One must represent a polypeptide or class of polypeptides that rifampicin elicits by interfering with cleavage of some of the normal polypeptides or by an aberration in synthesis. We found no indication of this in WR rifampicin particles (Fig. 7a), and therefore we have not further studied whole cytoplasmic extracts of WR-infected cells.

**DISCUSSION**

Rifampicin inhibits replication of WR and cowpox in HeLa-S3 cells. The decrease in rate of protein synthesis that occurs in the cytoplasm of WR-infected cells is accelerated by rifampicin.

The cowpox system differs in two respects: after an initial depression, there is no decrease in the rate of protein synthesis in the cytoplasm of cowpox-infected cells for at least 10 hr, and rifampicin does not markedly affect this rate of synthesis. In support of this point, we found that rifampicin had little effect on synthesis of the cowpox-induced late enzyme acid deoxyribonuclease. Our observations with cowpox virus do not support the suggestion of Ben-Ishai et al. (1) that a primary effect of rifampicin is to inhibit incorporation of amino acids into late viral proteins. At present, it is difficult to account for the difference between the response of cowpox and WR infection to rifampicin. It is conceivable that differences in the rates of replication of the two viruses in HeLa cells have something to do with the rifampicin response.

We do know that the rate of late messenger RNA synthesis and the time, postinfection, of peak late messenger synthesis are practically the
same in cowpox- or WR-infected cells (unpublished observations). There is no evidence from studies on induction of early or late enzymes (for an extensive list of references, see 4) that cowpox infection is asynchronous.

Inhibition of virus assembly caused by rifampicin does not necessarily lead to depression of viral protein synthesis as proposed for the WR system by Moss et al. (9). Rifampicin inhibition of RNA polymerase induction, at least in the cowpox system, is not due to a general reduction in the rate of late protein synthesis. Inhibition of RNA polymerase induction could be due to failure in formation of the necessary minimal subviral structure required for activity or, as suggested by McAuslan (3) and recently demonstrated (S. Dales, personal communications), binding of rifampicin to a soluble form of the enzyme.

Since rifampicin elicits a change in the poly-
peptide profile of cowpox-infected cells, its primary action might not be simply a block in assembly of normal components; there might be interference with polypeptide cleavage. Poxvirus strains that show differences in their response to rifampicin should be useful to determine the essential action of rifampicin and should provide information on the regulation of late events in replication. As in WR-infected cells, rifampicin inhibition of cowpox assembly is reversible; in the absence of further protein synthesis, this assembly is poor, and the resultant particles appear to be slightly denser than normal virions. It is clear from our data and from that of Moss et al. (9) that reversal of rifampicin inhibition is much more effective if there is concomitant protein synthesis. Since there is no marked rifampicin inhibition of cowpox-directed protein synthesis and all structural polypeptides are synthesized, we are led to the conclusion either that rifampicin inhibits synthesis of some unknown but essential maturation factor or that it binds tightly to some structural component which will jam assembly if not synthesized free from rifampicin. The most likely candidate for the latter proposition, by analogy with bacterial systems, would be the structural RNA polymerase.

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LITERATURE CITED