**Events in Vaccinia Virus-infected Cells Following the Reversal of the Antiviral Action of Rifampicin**

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**SUMMARY**

Infectivity titration, sedimentation analysis in sucrose gradients and electron microscopy have been used to study virus maturation following the reversal of the inhibition of vaccinia virus growth by rifampicin.

Electron-dense inclusions containing tubular structures develop in the cytoplasm of infected BHK21/C13 cells maintained in rifampicin, but the formation of immature and mature virus particles is prevented. The removal of rifampicin is followed by a rise of the virus infectivity. Spicule-covered membranes appear at the periphery of the inclusions and both immature and mature virus particles are seen. A proportion of the DNA synthesized in the presence of rifampicin is incorporated into particles and becomes resistant to deoxyribonuclease I. If protein synthesis is inhibited, spicule-covered membranes and immature particles appear but no mature particles are seen; the virus infectivity does not increase and the DNA remains susceptible to deoxyribonuclease I. It is suggested that rifampicin binds reversibly to a virus-specified protein, thereby preventing the formation of immature virus particles. Possible effects of rifampicin on the subsequent stages of virus maturation are discussed.

**INTRODUCTION**

Rifampicin inhibits the growth of bacteria by binding to and subsequently inhibiting DNA-dependent RNA polymerase (Wehrli *et al.* 1968; Sippel & Hartmann, 1968). The antibiotic also possesses antiviral activity against vaccinia and other poxviruses (Heller *et al.* 1969; Subak-Sharpe, Timbury & Williams, 1969). Studies on the mechanism of the antiviral effect have shown that rifampicin does not prevent virus RNA synthesis (Moss, Katz & Rosenblum, 1969a; McAuslan, 1969; Ben-Ishai *et al.* 1969), virus DNA synthesis (Subak-Sharpe *et al.* 1969; McAuslan, 1969; Ben-Ishai *et al.* 1969; Moss *et al.* 1969b), or early and late virus protein synthesis (Moss *et al.* 1969a, b; Subak-Sharpe *et al.* 1970) However, immature and mature virus particles are not formed in the presence of the drug (Moss *et al.* 1969b). The antiviral effect of rifampicin is reversible (Moss *et al.* 1969a; Subak-Sharpe *et al.* 1970). Moss *et al.* (1969b) also report that mature virus particles are produced in the absence of protein synthesis following the removal of rifampicin late in the growth cycle.

The experiments reported here have been concerned with analysing the events which occur after the removal of rifampicin between 13 and 17 hr after infection; in the absence of rifampicin, virus yield under one-step growth conditions approaches maximum level at these times (Subak-Sharpe *et al.* 1970).
METHODS

Materials. Rifampicin was a gift from Professor P. Sensi and colleagues of Lepetit S.P.A. (Milan) and Lepetit Pharmaceuticals Ltd, Slough, Buckinghamshire. Cycloheximide and puromycin were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Streptomycin was a gift from Dr R. G. Jacomb, Upjohn Ltd, Crawley, Sussex. [3H]-leucine of specific activity 250 mc/m-mole and methyl-[3H]thymidine of specific activity 20·2 c/m-mole were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Cell cultures. BHK 21 cells (clone 13) were grown as monolayers in 5 cm. plastic Petri dishes or 20 oz. glass bottles in Eagle's medium containing 10 % calf serum and 10 % tryptose phosphate broth. Coverslip cultures were prepared in Petri dishes.

Virus production and assay. Vaccinia virus (EVANS vaccine strain) and rabbit-pox virus (UTRECHT strain) were grown in monolayers of BHK cells. Estimates of infectivity were made by plaque assay using BHK cell monolayers with a liquid overlay in assays of vaccinia and an agar overlay in assays of rabbit-pox virus. Two days after infection the overlay was removed and the monolayers were stained with Giemsa stain.

Virus growth. Cell monolayers in Petri dishes were infected at an input multiplicity of 10 p.f.u./cell. After adsorption for 1 hr at room temperature the residual inoculum was removed, the cells were washed, and Eagle's medium containing 5 % calf serum together with the inhibitors under investigation was added. The cultures were then incubated at 37 °. Cells were harvested after removal of the medium by scraping into 0·2 ml. of 0·001 m-tris pH 9·0. Before titration of infectivity cells were disrupted by freezing and thawing followed by sonic treatment with a Dawe 250 W generator.

Sucrose density gradient centrifugation of methyl-[3H]thymidine-labelled DNA. Monolayers of BHK cells were infected with vaccinia virus at an input multiplicity of 10 p.f.u./cell and were maintained in medium containing 100 #g./ml rifampicin. The cells were exposed to a 2 hr pulse of methyl-[3H]thymidine (0·2 μc/ml) from 5 to 7 hr after infection and the rifampicin was removed 12 hr after infection. After incubation for a further 12 hr in the presence or absence of cycloheximide (300 #g./ml) the cells were harvested and disrupted as above. Samples were centrifuged at 2000 rev./min. for 5 min. to remove cell debris and 1 ml. portions of the supernatant fluids were again sonically treated for 3 min. and sedimented through 25 % to 60 % linear sucrose gradients in 1 mm-tris-HCl (pH 9·0) for 45 min. at 16,000 rev./min. using the Spinco SW 25·1 rotor at 5 °. Fractions of 13 drops were collected and 0·05 ml. from each was placed on a Whatman No. 1 filter paper disc (diameter 2·5 cm.). These were dried, washed three times with 5 % ice-cold trichloroacetic acid, twice with ethanol and once with ether and their radioactivity was then counted. Tris-HCl buffer (pH 7·5) and MgCl2 solution were added to the remainder of the fractions to a final concentration of 20 and 2 mm, respectively. These fractions were then incubated for 1 hr at 37 ° in the presence of 50 #g./ml deoxyribonuclease (Sigma DNase I from beef pancreas). Samples (0·05 ml.) were then placed on filter paper discs and radioactivity was measured after washing as described above.

Measurement of radioactivity. Coverslips and filter paper discs were placed in 10 ml. toluene-based scintillant and radioactivity was counted in a Nuclear-Chicago Mark I liquid scintillation computer (Model 6860). The efficiency of counting 3H on the filter paper discs was 12 %, on the coverslips 45 %.

Electron microscopy. For electron microscopy, BHK cells were grown in 20 oz. bottles; all other conditions of growth and infection with vaccinia virus were identical to those described above. The cells were scraped off the glass, the medium removed by centrifugation
and the cell pellet immediately fixed in glutaraldehyde followed by osmium tetroxide. The conditions of fixation and subsequent dehydration and embedding were as described by Goldman & Follett (1969).

Thin sections were cut on an LKB Ultrotome I, stained with uranyl acetate and lead hydroxide, mounted on uncoated grids and examined in a Siemens Elmiskop Ia operating at 80 kv.

**RESULTS**

The role of protein synthesis in the production of infectious virus which follows the reversal of rifampicin inhibition was examined using three inhibitors of protein synthesis: cycloheximide, streptovitacin and puromycin. Monolayers infected with vaccinia virus were maintained in medium containing rifampicin for up to 17 hr. The rifampicin was removed and replaced by medium containing the appropriate inhibitor. In some experiments the same inhibitor of protein synthesis was added to the medium 15 min. before removal of the rifampicin. In contrast to the results obtained by Moss et al. (1969b), the removal of rifampicin with subsequent inhibition of protein synthesis was not followed by a significant increase in virus infectivity (Fig. 1, Table 1). This result was obtained with each inhibitor of

![Graph](image)

*Fig. 1. Effect on growth of vaccinia virus of the removal of rifampicin 13 hr after infection (*) with subsequent inhibition of protein synthesis. ■—■, Rifampicin (100 μg./ml.) present; ○—○, rifampicin removed and replaced with normal medium; •—•, rifampicin removed and replaced with medium containing cycloheximide (300 μg./ml.).*
Table I. Effect of inhibitors of protein synthesis on virus growth after removal of rifampicin*

<table>
<thead>
<tr>
<th>Expt</th>
<th>Cells</th>
<th>Virus</th>
<th>Inhibitor of protein synthesis added after removal of rifampicin</th>
<th>Time of incubation (hr)</th>
<th>Infectivity (p.f.u./plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BHK</td>
<td>Vaccinia</td>
<td>None</td>
<td>0</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>6</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Streptovitacin 10 μg/ml.</td>
<td>6</td>
<td>$2.5 \times 10^4$</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cycloheximide 10 μg/ml.</td>
<td>6</td>
<td>$3.2 \times 10^4$</td>
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<tr>
<td>2</td>
<td>BHK</td>
<td>Vaccinia</td>
<td>None</td>
<td>0</td>
<td>$1.3 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>5</td>
<td>$1.6 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Puromycin 375 μg/ml.</td>
<td>5</td>
<td>$1.8 \times 10^6$</td>
</tr>
<tr>
<td>3</td>
<td>BHK</td>
<td>Rabbit-pox</td>
<td>None</td>
<td>0</td>
<td>$8.2 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>5</td>
<td>$1.8 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cycloheximide 300 μg/ml.</td>
<td>5</td>
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<tr>
<td>4</td>
<td>HeLa</td>
<td>Vaccinia</td>
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<td>0</td>
<td>$3.5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>6</td>
<td>$2.0 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cycloheximide 300 μg/ml.</td>
<td>6</td>
<td>$2.8 \times 10^4$</td>
</tr>
</tbody>
</table>

* Monolayers were infected with an input multiplicity of 10 p.f.u./cell and were incubated in medium containing rifampicin (100 μg/ml). At 17 hr after infection the medium was removed and replaced with fresh medium containing the appropriate inhibitor. Incubation was then continued for the time indicated.

protein synthesis. Similar results were obtained with BHK and HeLa cells; and also with vaccinia and rabbit-pox virus. In all experiments a significant increase in virus infectivity occurred after the removal of rifampicin and its replacement with normal medium (Fig. 1, Table I).

Incorporation of DNA into virus particles

Sucrose density gradient centrifugation of methyl-[3H]thymidine-labelled DNA from infected cells showed that DNA synthesis took place in the presence of rifampicin (Fig. 2a). Under these conditions the DNA remained at the top of the gradient and was not associated with virus particles which sedimented to \( \frac{1}{3} \) to \( \frac{1}{2} \) way down the tube (Joklik & Becker, 1964). As expected, a fast sedimenting methyl-[3H]thymidine-labelled component was obtained from similar preparations which had been incubated in normal medium following the removal of rifampicin (Fig. 2b). This component sedimented at the same rate as the fast moving component from infected cells maintained in normal medium throughout (Fig. 2d) and as virus particles purified by the method of Joklik (1962). The labelled DNA of the fast sedimenting component was resistant to deoxyribonuclease I, unlike the DNA at the top of the sucrose gradient which was destroyed by treatment with deoxyribonuclease I. (Fig. 3a) This evidence indicates that DNA synthesized in the presence of rifampicin is incorporated into virus particles following removal of the drug.

DNA synthesized in the presence of rifampicin was not incorporated into virus particles to any significant extent when inhibition of protein synthesis by cycloheximide followed the removal of rifampicin (Fig. 2c, 3b). Similarly, DNA synthesized in the presence of rifampicin was not incorporated into virus particles when puromycin was used as an inhibitor of protein synthesis in place of cycloheximide.
Antiviral action of rifampicin

Electron microscopy

A comparison of the process of infection of BHK 21/C13 cells with vaccinia virus in the presence and absence of rifampicin (100 μg./ml.) revealed three major differences. First, in the presence of rifampicin neither immature nor mature particles were seen. Secondly, cells infected in the presence of rifampicin were distinguished by characteristic clusters of electron dense (R1) inclusions present in the cytoplasm (Fig. 4). On removal of the drug, immature
particles developed from these inclusions (see below). They were thus identified as the sites of virus synthesis and corresponded to the 'factory' areas seen in the course of normal infection in the presence or absence of rifampicin (Cairns, 1960; Subak-Sharpe et al. 1969). The discrete R1 inclusions within a cluster varied in size; many R1 inclusions were completely surrounded by an irregular, wavy membrane; in others there were apparent gaps in this membrane (Fig. 5a). Some of the R1 inclusions (30 to 60% in these experiments) contained well-defined tubular structures in close-packed array (Figs. 5a, b). Centre-to-centre measurements of the tubes gave a mean diameter of 30 nm.; at higher magnification cross-sections of tubes showed a hexagonal subunit structure of about 10 nm. diameter.

The third major difference was the observation of a second type of inclusion (R2) within the factory areas of cells when rifampicin was present (Fig. 4). These R2 inclusions were smaller than the type described above, were very much denser and had no associated membrane. At higher magnifications the R2 inclusions showed a striated substructure (Fig. 5c). In the BHK cells examined here, R2 inclusions were rare.

Removal of rifampicin and incubation in normal medium for 1 hr produced marked alterations in the clusters of R1 inclusions. Immature particles were found within and around the factory areas and also in the process of forming from R1 inclusion bodies (Fig. 6). The characteristic spicule-covered membrane of the immature particles (Dales & Mosbach, 1968) appeared to form at several points on the periphery of each discrete R1 inclusion. From many observations it appears that these membranes gradually extend to enclose a standard volume of material from the R1 inclusion. A few mature particles were seen at this stage.

Fig. 3. Sucrose density gradient centrifugation patterns of deoxyribonuclease resistant methyl-[3H]thymidine-labelled DNA from vaccinia virus-infected cells. Fractions from the sucrose density gradients illustrated in Fig. 2 were treated with deoxyribonuclease I. (a) Rifampicin replaced with normal medium, (b) rifampicin replaced with cycloheximide.
Fig. 4. Electron micrograph of a thin section of a BHK 21/C13 cell 17 hr after infection with vaccinia virus. After infection the cells were incubated with medium containing 100 μg./ml. rifampicin. Numerous electron-dense, R1 (→) inclusions are apparent in this area of the cytoplasm. A few of the smaller, denser R2 (→→) inclusions are also present.
Fig. 5a. Electron micrograph of a thin section through a cluster of R1 inclusions. Rod-shaped structures can be seen in longitudinal and cross-section within the inclusions.

Fig. 5b. Enlargement of the R1 inclusion of Fig. 5a, illustrating a close-packed array of rods. The electron-transparency of the cores of the rods may indicate a tubular structure.

Fig. 5c. Enlargement of one of the R2 inclusions seen in Fig. 4 illustrating the striated sub-structure of these inclusions.
Fig. 6. Electron micrograph of a thin section of a BHK 21/C 13 cell infected with vaccinia virus and incubated for 15 hr in medium containing 100 μg/ml rifampicin. The rifampicin was then removed and replaced by normal medium for 1 hr. Numerous immature particles in various stages of formation are evident; the majority are at the periphery of the original R1 inclusions. No tubes are apparent within the inclusions, nor is there any peripheral membrane such as that in Fig. 4 and 5a.
Fig. 7. Electron micrograph of a thin section of a BHK 21/C13 cell infected with vaccinia virus and incubated for 17 hr in medium containing 100 μg./ml. rifampicin. At this time the rifampicin was removed and replaced by medium containing 375 μg./ml. puromycin. Incubation was continued for a further 6 hr. A pronounced alteration is evident in the appearance of the R1 inclusions compared to those in Fig. 4; the situation is more comparable to that in Fig. 6 with immature particles again forming at the periphery of the original R1 inclusions.
Incubation for 2½ hr after the removal of rifampicin led to the formation of many mature particles. After either 1 or 2½ hr incubation following the removal of rifampicin, no sign of the original peripheral membrane of the R I inclusion remained and no tubular structures were seen within the altered inclusions.

Removal of rifampicin in the presence of cycloheximide and puromycin resulted also in changes in the R I inclusion bodies. Numerous immature particles formed around the periphery of each inclusion (Fig. 7); few were completely formed and none were mature. Measurement of [3H]L-leucine incorporation by infected cells indicated that cycloheximide and puromycin at the concentrations used depressed protein synthesis by more than 99% (Table 2).

Table 2. Effect of inhibitors of protein synthesis on incorporation of [3H]leucine into coverslip cultures of BHK cells

<table>
<thead>
<tr>
<th>Inhibitor of protein synthesis</th>
<th>Leucine incorporation: [3H]counts/min./coverslip</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Expt 1</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>10</td>
</tr>
<tr>
<td>Puromycin</td>
<td>4</td>
</tr>
</tbody>
</table>

Cultures were infected with an input multiplicity of 10 p.f.u./cell and were incubated in rifampicin (100 μg/ml.) for 16 hr. The rifampicin was then removed and incubation was continued for 5 hr in medium containing [3H]-leucine (1 μCi/ml.) or no inhibitor. Coverslip cultures were then washed twice in phosphate-buffered saline, pH 7.2, fixed in formol saline, washed twice with ice-cold 10% trichloroacetic acid, washed with water, rinsed with absolute ethanol and dried. Radioactivity was then counted.

DISCUSSION

These electron microscope studies show that in BHK 21/C13 fibroblasts neither mature nor immature particles of vaccinia virus are made in the presence of rifampicin; this is in accord with the previous findings of Moss et al. (1969b). In their HeLa cell suspension cultures rifampicin had a similar effect on virus growth and, as in our system, removal of the drug resulted in the appearance of immature and mature virus particles. Their HeLa cells showed electron dense bodies similar to our R I inclusions but tubular structures were not mentioned. The significance of the tubular structures that we observe in some R I inclusion bodies is unknown. Tubular structures of much smaller diameter are known to be present on the surface of vaccinia virus particles (Westwood et al. 1964). These appear to be more comparable to constituent subunits of the tubular structures in R I inclusions. It is possible that the R I tubes are a by-product not normally made in large amounts and are formed in the absence of virus maturation from some of the virus products known to be synthesized in the presence of rifampicin.

The R 2 inclusions seen by us in BHK cells were not described by Moss et al. (1969b) for the HeLa cell system but were observed by S. Dales (personal communication, 1969) in mouse L 2 cells infected with the H 1D strain of vaccinia virus in the presence of rifampicin.

It can be concluded from the reversal studies that rifampicin blocks the formation of immature particles. In particular, the unique spicule-covered outer membranes of the immature forms are never seen in infected cells in the presence of rifampicin. After rifampicin has been removed spicule-covered membranes can form without further protein synthesis. It appears that the constituents of both membrane and spicules are made in the presence of rifampicin.
and that the drug acts by preventing the assembly of at least these constituents into the characteristic immature particles.

Our results clearly show that two distinct processes are required for the production of mature virus following the removal of rifampicin. The first event, which occurs in the absence of protein synthesis, leads to the formation of immature virus particles. At this stage, virus DNA remains susceptible to the action of deoxyribonuclease I. The subsequent events, which lead to the production of mature infectious virions containing deoxyribonuclease I-resistant DNA, require de novo protein synthesis. These findings differ from those obtained by Moss et al. (1969b), who reported that a limited production of mature infectious virus particles occurred in the absence of protein synthesis following the removal of rifampicin. They are in agreement with results obtained by S. Dales (personal communication, 1969) using streptovitacin in L2 cells infected with the IHD strain of vaccinia virus.

The isolation of rifampicin-resistant mutants of vaccinia virus indicates that the antiviral effect of the drug follows a specific interaction with a single virus protein (Subak-Sharpe et al. 1970). Present results indicate that this protein is involved in the formation of immature virus particles. The formation of these particles in the absence of protein synthesis, when rifampicin has been removed, suggests that the binding of rifampicin to the protein is freely reversible. This contrasts with the situation in bacterial systems, in which rifampicin forms a very stable complex with the DNA-dependent RNA polymerase (Wehrli et al. 1968).

The requirements for de novo protein synthesis in the production of mature virus particles which follows the removal of rifampicin may be interpreted in several ways. It is possible that one, or more than one, of the proteins required for the subsequent final stages of virus assembly is produced in the presence of rifampicin but is labile and that continuous protein synthesis is therefore needed for the production of virus particles. Alternatively, rifampicin could prevent, directly or indirectly, the production of such a protein or proteins. For example, rifampicin may block the transcription of the mRNA coding for these proteins, or controls at the level of transcription or translation may only allow the formation of such proteins after the assembly of immature virus particles. At present we cannot distinguish between these possibilities.

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REFERENCES


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