Inhibition of Human Cytomegalovirus by Rifampin

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SUMMARY

Replication of human cytomegalovirus (CMV) was inhibited by 50 μg/ml of rifampin. Nevertheless, a number of functions of CMV were still expressed in the presence of rifampin, including early cell rounding, and the development of immuno-fluorescent antigen, haemadsorption antigen, complement-fixing antigen and precipitin antigens. If rifampin was kept in the culture medium for longer than 48 h, infectious virus was not synthesized, but removal of rifampin resulted in restoration of virus titre within 24 h. In parallel with the restoration of infectivity, removal of the drug resulted in a sharp increase in macromolecular synthesis, first RNA and then virus DNA. The results suggest that rifampin blocks a stage in the production of m-RNA species.

INTRODUCTION

The antibiotic rifampin is active against several DNA viruses, particularly poxviruses and adenoviruses (Subak-Sharpe, Timbury & Williams, 1969), but is without activity against herpes simplex viruses types 1 and 2. Nevertheless, rifampin has been reported to inhibit another herpes group virus, the human cytomegalovirus (CMV; Halsted, Minnefor & Lietman, 1972). This paper describes the effect of rifampin on virus synthesis, virus DNA synthesis and certain virus antigens of human CMV.

METHODS

Virus. The Towne strain (Furukawa, Fioretti & Plotkin, 1973) of CMV was used throughout the study. It had been continuously propagated in WI-38 cell culture for more than 100 passages in our laboratory.

Cell culture. WI-38 human diploid fibroblasts were obtained from Flow Laboratory and from Dr L. Hayflick, Stanford University. Cells were used at passage levels between the twentieth and the twenty-eighth generations. The cells were grown in Eagle’s minimal essential medium (MEM) with 10% foetal calf serum and maintained with 2% foetal calf serum.

Infectivity assays. Tube cultures of WI-38 cells were inoculated and then incubated in a stationary position at 37 °C. The end point of infectivity determined by induction of c.p.e., was read 14 days after infection. The titre in TCD₉₀ was calculated by the Reed & Muench method (1938).

Haemadsorption. The method used was essentially the same as described by Watkins

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Briefly, sheep red blood cells (RBC) were sensitized by adding 0.01 ml rabbit anti-sheep RBC serum to 10 ml of a 4% suspension of sheep RBC in MEM. After incubation at 37 °C for 1 h and 3 washes in phosphate buffered saline (PBS) at pH 7.4, the sensitized cells were resuspended in MEM to a final concentration of 1%. At various intervals post infection (p.i.), the coverslips were washed and 1% sensitized sheep RBC were added. After incubation for 2 h at 37 °C, the coverslips were washed 3 times with PBS, stained with Giemsa and then examined microscopically.

Precipitin and complement-fixing (CF) antigens. Tests for precipitin and CF antigen determination were as described previously (Furukawa et al. 1973).

Incorporation of radioactive precursor. Cell monolayers in Falcon Petri dishes (3 cm diam.) were infected with CMV. After virus adsorption at 37 °C for 1 h, the cultures were washed with maintenance medium, and then further incubated under 3 ml of medium. Uninfected controls were treated similarly. At different times after infection, three dishes from each group were pulse labelled for 1 h with 5 μCi of [3H]-thymidine (sp. act. 20 Ci/mmole) per ml, followed by three washes with cold PBS. The cells were lysed by adding 0.1 N-sodium hydroxide. To label RNA, [3H]-uridine (40μCi/ml, New England Nuclear, Boston, Mass.) was added to cultures for 2 h pulses. After labelling, the cultures were washed then lysed with 1% sodium dodecyl sulphate in PBS. The sample was precipitated with 10% trichloroacetic acid (TCA) and the precipitate was collected on nitrocellulose filters, washed with 5% TCA, dried and then counted in toluene-omnifluor (New England Nuclear, Boston, Mass.). Radioactivity determinations were made in a Beckman liquid scintillation counter.

Separation of cellular and virus DNA. Cells in 10 cm Petri dishes were labelled with [Methyl-3H]-thymidine as described above. Labelled cells were lysed in TE (0.05 M tris-HCl, pH 9.0, 0.002 M-EDTA) containing 1% SDS, and digested overnight with pronase (1 mg/ml). After digestion, DNA was extracted by phenol, precipitated by alcohol, dissolved in 0.2 ml of TE, mixed well with 4.8 ml of caesium chloride (CsCl) solution in TE (1.700 g/ml), and centrifuged for 68 h at 33000 rev/min in a Spinco SW 50.1 rotor.

Two drop fractions were collected from the bottom of the centrifuge tube and acid-insoluble radioactivity was measured. The radioactivity appearing in the density region of 1.718 was considered virus DNA and that of 1.700 considered cellular DNA. The incorporation of [3H]-thymidine by uninfected cells in the 1.718 density region was considered to be background.

Rifampin. Rifampin (Dow Chemical Company, Zionsville, Ind.) was dissolved in MEM and sterilized through a Nalgene Filter unit (pore size 0.20 μm, Sybron Corp., Rochester, N.Y.). Fresh solutions were prepared for each experiment.

Chemicals. [Methyl-3H]-thymidine was purchased from New England Nuclear Corp. (Boston, Mass.). Cycloheximide was from Nutritional Biochemical (Cleveland, Ohio) and FUDR from Sigma Chemical Co. (St Louis, Mo.).

RESULTS

Cultures were infected at a multiplicity of 10 TCD50 per cell. After 1 h adsorption the cells were washed 2 times and then covered with maintenance medium with or without rifampin. At 4 days p.i., the cells were trypsinized and sonicated for 2 min, and clarified sonicates were assayed for infectivity. Cells on coverslips infected in parallel were stained with Giemsa for cytopathology. The results are shown in Table 1. Virus replication was inhibited at concentrations of 50 μg/ml or over. Inclusion body formation was also inhibited, whereas the early c.p.e. that appears between 6 to 12 h p.i., was not affected even
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Table 1. Effects of rifampin on the growth of CMV in WI-38 cells

<table>
<thead>
<tr>
<th>Concentration of rifampin in medium (μg/ml)</th>
<th>Infectivity*</th>
<th>Early c.p.e.</th>
<th>Cells with inclusion bodies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>&lt; 10^1:9</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>&lt; 10^1:9</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>10^3:5</td>
<td>+</td>
<td>70</td>
</tr>
<tr>
<td>10</td>
<td>10^7:5</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>10^7:5</td>
<td>+</td>
<td>100</td>
</tr>
</tbody>
</table>

* Infectivity TCD_{50}/0:2 ml at 4 days p.i.

Table 2. Effects of rifampin on CMV complement-fixing (CF), precipitin and haemadsorption antigens 4 days post-infection

<table>
<thead>
<tr>
<th>Treatment rifampin</th>
<th>Infectivity*</th>
<th>Precipitin antigens</th>
<th>Haemadsorbing cells</th>
<th>CF† antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td>&lt; 10^1:9</td>
<td>1:2</td>
<td>82%</td>
<td>1:32</td>
</tr>
<tr>
<td>None</td>
<td>10^7:5</td>
<td>1:4</td>
<td>67%</td>
<td>1:32</td>
</tr>
</tbody>
</table>

* Infectivity TCD_{50}/0:2 ml.
† Complement-fixing activity.

by the highest concentration of rifampin tested (Table 1) in either treated or non-treated cells.

The influence of rifampin on production of CF antigen, precipitin antigen, fluorescent antigen and haemadsorption antigen was tested. Petri dish cultures containing coverslips were used for fluorescent and haemadsorption tests. Large (10 cm diam.). Petri dish cultures harvested at 5 days p.i. were used for determination of precipitin and CF antigens. In the immunofluorescence study, coverslip cultures were fixed at various intervals p.i. At 3 h p.i., a granular form of fluorescence was seen in the cytoplasm. At 6 h p.i., a nuclear and perinuclear fluorescence was seen that continued through the 5 day observation period.

All the antigens detected in infected cultures were also detected in rifampin-treated cultures despite the inhibition of production of infectious virus (Table 2). The precipitin titre, however, was lower than in control cultures.

CMV has an eclipse period of 48 h (Plummer et al. 1969; Iwasaki et al. 1973). To determine whether rifampin affects the events that take place during this eclipse period, cells were infected at a multiplicity of 50:1. After 1 h adsorption the medium was replaced with maintenance medium containing 100 μg/ml of rifampin. At various intervals p.i., cultures were washed free of rifampin, maintained with ordinary media, and the supernatant fluid and cell associated virus assayed. The results are shown in Fig. 1. If rifampin was removed from CMV-infected cells at any time up to 48 h p.i., the time of the onset of virus DNA synthesis (Furukawa et al. 1973), virus growth was unaltered. When rifampin was kept in the cultures longer than 48 h p.i., infectious virus was not synthesized until the removal of rifampin. If rifampin was removed at 96 h p.i. there was complete restoration of virus titre within 24 h (Fig. 1).

To determine whether protein inhibition influenced virus replication after removal of rifampin at a late stage of infection, an additional experiment was performed. At 72 h p.i., rifampin was removed, cycloheximide (20 μg/ml) was added to the medium and infectivity
Fig. 1. Effect of rifampin withdrawal on the growth of CMV. The rifampin was removed at time intervals indicated by arrows. Virus titration was not carried out below 10^1 TCD₉₀ (diagonal lines). ——, no rifampin; ——, rifampin removed at 24 h; ——, rifampin removed at 48 h; ——, rifampin removed at 72 h; ——, rifampin removed at 96 h.

Fig. 2. Effect of addition of cycloheximide to CMV-infected cultures treated with rifampin up to 72 h, at which time rifampin was removed. Virus titration was not carried out below 10^1 TCD₉₀ (diagonal lines). ——, no rifampin; ——, rifampin 100 μg/ml for 72 h; ——, cycloheximide 20 μg/ml added at time of rifampin removal.

was assayed. In control cultures, virus titres increased rapidly, reaching a titre one log lower than in non-treated cultures; infectious virus synthesis was inhibited within 6 h after removal of rifampin (Fig. 2) in the cycloheximide-treated culture. Thus the rifampin-treated cells needed additional protein synthesis to make infectious virus.

Electron microscopical studies demonstrated no intranuclear particles in CMV-infected WI-38 cells in the presence of rifampin up to 96 h p.i. Within 12 h after the withdrawal of
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Fig. 3. Effect of rifampin (100 µg/ml) on [3H]-thymidine incorporation in CMV-infected WI-38 cells: O—O, CMV without rifampin; ⋅⋅⋅⋅⋅⋅, CMV with rifampin; ..., control uninfected cells.

Fig. 4. Effect of rifampin (100 µg/ml) on [3H]-uridine incorporation in CMV-infected WI-38 cells: O—O, CMV without rifampin; ⋅⋅⋅⋅⋅⋅, CMV with rifampin; ..., control uninfected cells.

rifampin, a few particles were seen in the nucleus. By 24 h after withdrawal of the drug, the process of envelopment at the nuclear membrane and the presence of mature virus particles in intracytoplasmic vacuoles were detected.

At various intervals, infected cells treated with rifampin were pulse-labelled for 1 h with [methyl-3H]-thymidine and the incorporation of radioactive label was expressed as percentages of control. As shown in Fig. 3, the uptake of thymidine in rifampin-treated cultures did not exceed that in uninfected control cultures, while in non-treated infected cultures thymidine uptake began to increase at 48 h.

To determine the course of RNA metabolism under the influence of rifampin, a similar experiment was performed after pulse-labelling with tritiated uridine. As shown in Fig. 4, the expected stimulation of cellular RNA synthesis produced by CMV infection was completely abolished by the presence of 100 µg/ml of rifampin (Tanaka, Furukawa & Plotkin, 1975).

In another experiment, rifampin was removed at 72 h p.i., and infected cultures were labelled with [3H]-thymidine. Twenty-four h after removal of rifampin, extracted labelled
Table 3. Analysis of DNA* of rifampin-treated†, CMV-infected cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus</th>
<th>Cellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMV-infected cells</td>
<td>Control</td>
</tr>
<tr>
<td>Rifampin</td>
<td>9050</td>
<td>110</td>
</tr>
<tr>
<td>No rifampin</td>
<td>7342</td>
<td>102</td>
</tr>
</tbody>
</table>

* In terms of cts/min of [3H]-thymidine incorporated 24 h after removal of rifampin.
† Rifampin removed 72 h p.i.

DNA was subjected to isopycnic sedimentation in CsCl for 72 h at 20 °C. Parallel determination of density of DNA from purified CMV gave a buoyant density of 1.716 for virus DNA, whereas DNA from control WI-38 cells had a buoyant density of 1.700. The peak of thymidine incorporation after withdrawal of the drug corresponded in sedimentation with that of virus DNA (Table 3). These results suggested that virus DNA was not synthesized in the presence of rifampin.

A similar withdrawal experiment was performed using [3H]-thymidine and [3H]-uridine labels to define the onset of both total RNA and DNA synthesis after rifampin removal. Fig. 5 shows that 2 h after removal there was a slight increase of RNA synthesis, and by 4 h a definite increase. DNA synthesis did not begin until 14 h after removal of rifampin.
DISCUSSION

Rifampin inhibited CMV growth but did not inhibit events expressed in the early period of the infectious cycle.

The activity of rifampin against other viruses has been demonstrated by several authors (Becker et al. 1969; Ben-Ishai et al. 1969; Diggelmann & Weissman, 1969; Moss et al. 1969; Engle, Lasinski & Gelzer, 1970). The mechanism of antiviral activity was extensively studied in vaccinia infection and the conclusions were that either rifampin binds to a sensitive polypeptide in the manner conceived for its effect on bacterial RNA polymerase and prevents the formation of an initiation complex or that it interferes with the function of only one of two or more RNA polymerases that may be induced by the virus (Pogo, 1971). Although it has been shown that vaccinia virus has an RNA polymerase in the virus particle (Kates & McAuslan, 1967), it is uncertain whether herpes viruses contain their own RNA polymerase or whether they use that of the host cells.

Since herpes simplex was insensitive, whereas CMV and Varicella-Zoster virus were sensitive to rifampin (Furukawa, Tanaka & Plotkin, 1974), the possibility that the latter viruses have intrinsic RNA polymerase should be studied. Rifampin sensitivity may provide a differential basis for distinguishing subgroups of herpes viruses.

Moss et al. (1969) concluded that rifampin interferes with the assembly of vaccinia DNA and protein into virus particles at a stage in the formation of the virus envelope. They demonstrated that in the presence of rifampin no virus was detected and virus DNA remained in a DNase-sensitive form.

In our experiment, we failed to demonstrate any form of virus DNA replication in the presence of rifampin by means of radioactive thymidine incorporation and DNA analysis. Although infectious virus was demonstrated within 6 h after rifampin was removed, the incorporation of thymidine only began to increase at 14 h after rifampin's removal. This discrepancy may result from the greater sensitivity of the infectivity assay over that of the measurement of thymidine incorporation. We also showed that incorporation of uridine into RNA resumed at least 10 h prior to the recovery of DNA synthesis; rifampin, therefore, may interfere with the synthesis of specific classes of m-RNA in the CMV infectious cycle.

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REFERENCES


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