Effect of Actinomycin D on Growth of Rubella Virus in Tissue Cultures

By WILNA A. WOODS AND F. C. ROBBINS

Department of Pediatrics, Cleveland Metropolitan General Hospital, and Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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SUMMARY

Multiplication of rubella virus in primary and continuous-line African green-monkey kidney cells was delayed by addition of low doses of actinomycin D to the culture medium before or at the time of infection. Addition of the drug 2 hr after infection was less effective. However, virus replication became normal after 3 to 8 days, although cellular RNA synthesis in drug-treated control cultures continued to be inhibited.

INTRODUCTION

Cultures of primary African green-monkey kidney cells (GMK) (Parkman, Buescher & Artenstein, 1962) or the continuous line of African green-monkey kidney cells (GMK, AH-1) (Gunalp, 1965) can support the slow multiplication of rubella virus for long periods with little or no cytopathic effect. Interference with the cytopathic effect of a challenge virus allows detection of rubella virus in GMK: however, the interfering factor in rubella-infected GMK does not appear to be classical interferon (Parkman et al. 1964). The present study was begun to determine if actinomycin D would enhance the virus yield and/or the cytopathic effect in the rubella-infected tissue culture, as reported for the Chikungunya virus + chick embryo cell system by Heller (1963). However, it was found that the drug delayed, but did not completely inhibit, rubella virus replication in both GMK and GMK, AH-1 cells when added in low concentrations before or at the time of infection. These findings agree with those of others (Barry, Ives & Cruickshank, 1962; Barry, 1964) for several viruses of the myxovirus group and for rubella virus according to a preliminary report by Maasab (1966); but Maes et al. (1966) did not find any inhibition of rubella virus when much larger doses of actinomycin D were added after the eclipse phase of rubella virus multiplication in RK-13 or GMK cells.

METHODS

Virus and virus titration. The rubella virus used in this study was obtained from Dr A. D. Heggie. It had been passed several times in both GMK and GMK, AH-1 cells. The virus pool used as inoculum was prepared in GMK, AH-1 cells and had a titre of $10^{5.5}$ 50% tissue culture interfering doses (TCID$_{50}$) per ml., calculated by the method of Reed & Muench (1938). All titrations were performed in GMK by methods previously described (Woods et al. 1966).
**Tissue culture.** Primary GMK cultures were grown in Eagle's basal medium with glutamine and 2% (v/v) foetal calf serum (BME-FCS). GMK, AH-1 cells were grown in Puck's medium (Woods et al. 1966). Both cells were maintained in BME-FCS after virus inoculation. Monolayer cultures in \(16 \times 125\) mm. screw-capped culture tubes were used for virus growth and RNA synthesis experiments. Cultures for acridine orange staining were prepared on sterile \(1 \times 3\) in. microscope slides (Cairns, 1960). Three sterile glass rings (10 mm. inside diameter) were sealed to each slide with a sterile mixture of 3 parts histowax to 1 part petroleum jelly; four slides were placed in a \(150\) mm. plastic Petri dish. A half ml. cell suspension was placed inside each ring, and the cultures were incubated at \(37^\circ\) in an atmosphere of 5% CO\(_2\) in air.

Cell counts were made on representative monolayer cultures. These were trypsinized with \(0.05\%\) trypsin (Difco 1:250) in Hanks's balanced salt solution, pH 7.8 to 8.0. The resulting cell suspension was counted in a haemocytometer. The number of cells per culture for both GMK and GMK, AH-1 was \(4 \times 10^5\) in tube cultures and \(3 \times 6 \times 10^4\) in ring cultures.

**Actinomycin D.** The drug was supplied by Merck, Sharp and Dohme, West Point, Pennsylvania. A stock solution containing 100 µg./ml was prepared in BME-FCS and was diluted in the same medium to obtain final concentrations of 0.05, 0.1 or 0.2 µg./ml. One ml. of actinomycin D solution was added to tube cultures or 0.5 ml. to ring cultures immediately before inoculation with virus, unless otherwise stated. Untreated cultures were changed with fresh BME-FCS at the same time. The cultures exposed to actinomycin D were protected from light during incubation.

**Uridine incorporation.** Synthesis of cellular RNA was estimated by measuring the amount of \([\text{H}]\)uridine (specific activity 7.2 µc/m-mole) incorporated into the cold trichloracetic acid insoluble cell fraction. The \([\text{H}]\)uridine, 0.25 µc, in 0.1 ml. BME, obtained from New England Nuclear Corporation, Boston, Massachusetts, was added without medium change to cultures to be examined for uridine incorporation. 5'-Fluorodeoxyuridine (Hoffman-LaRoche, Inc., Nutley, New Jersey) was added at the same time to a final concentration of \(10^{-4}\)M to inhibit incorporation of the label into DNA. The supernatant fluid was removed after 90 min. incubation at \(37^\circ\), and the cells were washed 4 times with 5 ml. cold \(0.85\)% NaCl. The cultures were extracted \textit{in situ} 3 times with cold 5% (w/v) trichloracetic acid, washed once each with 5 ml. absolute ethanol, ethanol+ether mixture (3:1) and absolute ether. The cellular residue was dissolved in 1 ml. m-hyamine hydroxide. The hyamine extracts of 4 replicate cultures were pooled, and 0.2 ml. of this pool was added to 18 ml. scintillation fluid and counted in a Nuclear Chicago liquid scintillation counter. The scintillation fluid consisted of 12.5 g. PPO (2,5 diphenyloxazole), 0.3125 g. POPOP (1,4-bis-(2-(5-phenyloxazolyl)) benzene), 125 g. Naphthalene, 1000 ml. \(p\)-dioxane. At least 99% of the cold trichloracetic acid-insoluble label was ribonuclease-sensitive, but deoxyribo-nuclease-insensitive.

**Acridine orange staining.** The acridine orange staining technique of Niven (1959) was used to estimate both the proportion of infected cells and the extent of inhibition in these cells in cultures in which RNA synthesis was inhibited by actinomycin D.
RESULTS

The effect of actinomycin D on the growth of the rubella virus

Since maximum titres of rubella virus are not reached in tissue culture for 5 to 7 days, this study was limited to the effect of those concentrations of actinomycin D which allowed cultures to be observed for at least a week. Tube cultures with or without actinomycin D in the maintenance medium were inoculated with rubella virus at an input multiplicity of approximately 0.1. The drug was not removed during the experiment. At intervals, the supernatant fluid of four cultures was harvested and pooled. The cells were washed 4 times with BME-FCS, 1 ml. of fresh medium was added to each tube, the cultures were quickly frozen and thawed once, and the fluids were pooled. All harvests were stored at -85°C.

Table 1. Growth of rubella virus in GMK in the presence of actinomycin D

<table>
<thead>
<tr>
<th>Actinomycin D (µg./ml.)</th>
<th>Rubella virus: log. TCID 50/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>0.00</td>
<td>1.5</td>
</tr>
<tr>
<td>0.05</td>
<td>Neg.</td>
</tr>
<tr>
<td>0.10</td>
<td>Neg.</td>
</tr>
<tr>
<td>0.20</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

Actinomycin D was added at time of infection.

Table 2. Growth of rubella virus in GMK, AH-1 cells in the presence of actinomycin D

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Actinomycin D (µg./ml.)</th>
<th>Rubella virus: log. TCID 50/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>1</td>
<td>0.00</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>0.00</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>Neg.</td>
</tr>
<tr>
<td>3</td>
<td>0.00</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

Actinomycin D added at time of infection.

Growth curves of rubella virus were constructed (Tables 1, 2). Since at any given time after new virus had appeared in either treated or control cells, the amounts of cell-associated virus were about the same as the virus in the supernatant fluid, only titres in the supernatant fluid are reported in the tables. Concentration of 0.1 or 0.2 µg./ml. actinomycin D in the medium caused a delay in the appearance of demonstrable virus in both GMK and GMK, AH-1 cultures; 0.05 µg./ml. also delayed virus multiplication in GMK, but was without effect in GMK, AH-1. However, the virus-synthetic ability of all drug-treated cultures was recovered, and these cultures later produced virus at titres comparable to those in the controls.

The time of addition of the drug relative to virus inoculation was important. Actinomycin D, 0.1 µg./ml. was added to cultures of GMK, AH-1 cells at the following
times: 3 hr before, at the same time as, or 2 hr after virus inoculation. Adsorption of the inoculum was allowed to take place for 2 hr at 37° before the initial samples were obtained, when 99% of the input virus was no longer detectable in the supernatant medium. Neither the virus inoculum nor the actinomycin D was subsequently removed (Fig. 1). Delay in viral multiplication was most noticeable when the drug was added 3 hr before or at the time of virus inoculation; the titre in these cultures did not attain that of the untreated controls until day 7 or day 8. When the virus was inoculated 2 hr before the drug was added, however, the virus titre of the treated cultures was lower than that of the controls on day 1, but had reached the same titre as the controls on day 3.

Fig. 1. Growth curves of rubella virus in the presence of 0.1 μg./ml. Actinomycin D added to cultures of GMK, AH-1 cells 3 hr before (■), at the time of (■) or 2 hr after inoculation (○), of 10 TCID₅₀ virus. ○, Control.

The effect of actinomycin D on incorporation of [³H]uridine into cellular RNA

The inhibitory action of actinomycin D on incorporation of [³H]uridine into cellular RNA was employed to ascertain if the concentrations of actinomycin D employed in the study were effective in inhibiting cellular RNA synthesis and if the delayed initiation of virus production was associated with recovery of cellular RNA synthesis. Cultures of GMK and GMK, AH-1 cells were exposed to actinomycin D, incubated, and extracted as described in methods (Fig. 2, 3). Early decrease in RNA synthetic ability was indicated by inhibition of [³H]uridine incorporation during the first 90 min. of drug exposure (i.e. [³H]uridine added at the same time as actinomycin D). Some degree of inhibition of RNA synthesis by actinomycin D was noted within this time; except that in GMK, AH-1 cultures treated with 0.05 μg./ml. viral multiplication was also not affected (Table 2). However, the degree of initial inhibition of RNA synthesis did not appear to affect the time of recovery of viral multiplication. Furthermore, gross recovery of cellular RNA synthetic ability was not necessary for recovery of viral productive capacity in GMK, AH-1 cells. Although virus was being produced in all cultures by the third day, the inhibition of RNA synthesis increased
Effect of actinomycin D on rubella virus

within this period with the exception of those cultures treated with 0.2 \( \mu \text{g.}/\text{ml.} \) actinomycin D, in which the inhibition was essentially complete within the first 90 min.

The effect of actinomycin D on RNA content of tissue culture cells

It was important to determine whether or not all cells were susceptible to the action of actinomycin D, since drug-resistant cells might have been the site of subsequent

![Graph](image)

**Fig. 2.** Incorporation of \([\text{H}]\text{uridine}\) into cellular RNA (cold trichloracetic acid-insoluble cell fraction) of GMK cells in the presence of actinomycin D. \([\text{H}]\text{uridine}, 0.25 \mu\text{c},\) was added to cultures at the same time as the drug (○) or 5 hr later (■); incorporation proceeded for 90 min. at 37° before harvest and extraction with cold 5% TCA.

virus multiplication. The bright red RNA colour of both GMK and GMK, AH-1 cells stained with acridine orange was noticeably diminished in only 10% to 25% of the cells after 1 to 2 days treatment with 0.05 \( \mu\text{g.}/\text{ml.} \) actinomycin D; reduction of nucleolar staining preceded that of cytoplasmic staining. Recovery of both nucleolar and cytoplasmic staining was evident in 4 days. In contrast, 0.1 \( \mu\text{g.}/\text{ml.} \) actinomycin D caused marked reduction of staining in all cells within 24 to 48 hr. Nucleolar RNA staining was again affected first, followed by reduction of cytoplasmic staining during the
second 24 hr of exposure, until all cells had uniformly low levels of staining. No increased staining intensity was noted for at least 8 days. Treatment with actinomycin D at a concentration of 0.2 µg./ml. completely destroyed both nucleolar and cytoplasmic RNA staining in all cells within 18 hr; no recovery of RNA staining was noted for at least 8 days. Therefore, while the effect in cells treated with 0.05 µg./ml. actinomycin D was equivocal, all cells in cultures treated with 0.1 or 0.2 µg./ml. were equally affected and supported the replication of virus in the face of sustained suppression of cellular RNA synthesis.

![Graph showing incorporation of [3H]uridine into cellular RNA](image)

**DISCUSSION**

The multiplication of influenza virus was shown by Barry et al. (1962) to be inhibited by small doses of actinomycin D; Barry (1964) also demonstrated similar inhibition of fowl plague virus. However, the replication of another myxovirus, Newcastle disease virus, was reported by these authors to be unaffected even by much larger doses of the drug. Temin (1963) found a similar inhibitory effect of actinomycin D on Rous sarcoma virus in chick embryo cell cultures. Both Barry (1964) and Temin (1963) demonstrated a concomitant inhibition of cellular RNA synthesis.

The results of the experiments reported here agree with those of Maasab (1966) that small doses of actinomycin D inhibit the growth of rubella virus. However, this
Effect of actinomycin D on rubella virus

inhibition was not complete, and normal amounts of virus were found in drug-treated cultures after a 3- to 8-day delay. Although one-step growth experiments were not feasible with the inoculum used in these experiments, it was apparent that drug treatment of the cells was more effective when it was applied before initiation of the viral eclipse phase. Whether the inhibition is due to delayed coding for virus-directed synthesis in the initial as well as subsequent cycles or to reduced production of mature virus throughout the growth period could not be determined from the data presented here. The one-step growth experiments of Maes et al. (1966) also implicate an early step in viral multiplication as the target of inhibition, since these authors found no inhibition of viral multiplication when high concentrations of the drug were added to rubella-infected RK-13 or GMK cells at the end of the eclipse phase or during the exponential growth period.

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REFERENCES


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