Effects of Actinomycin D on Early Steps of Replication in vitro of Murine Sarcoma Virus (Moloney)

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SUMMARY

The sensitivity to actinomycin D of the early steps of the in vitro replication of murine sarcoma virus (Moloney) (M-MSV) was investigated by treating infected mouse cells with the antibiotic (0.25 μg/ml) for 6 h periods at several times after infection. The results obtained indicate that M-MSV production is inhibited when actinomycin D is added within 2½ h of infection.

The sensitivity to actinomycin D of the replication of murine RNA tumour viruses has been demonstrated by Duesberg & Robinson (1967) and Bases & King (1967) using an established cell line continuously propagating Rauscher leukaemia virus, and by Buck & Bather (1969) using mouse embryo cells acutely infected with the Moloney strain of murine sarcoma virus (M-MSV). The results obtained by the latter authors suggested that the initiation of the cycle of replication of M-MSV was inhibited irreversibly by this antibiotic. However, the duration of the early actinomycin D-sensitive period was not determined and this particular point has been investigated.

Cell line 8828, derived from BALB/c mouse embryo (Bernard et al. 1968), was used in all experiments. M-MSV stocks were prepared from BALB/c mouse tumour extracts and titrated on 8828 cells. Virus stocks contained both murine sarcoma virus (MSV) and murine leukaemia virus (MLV) (Bernard et al. 1968).

Cells were seeded in Kahn tubes or in Falcon Petri dishes (50 mm diam.) at an initial concentration of 3 x 10⁴ cells/tube and 6 x 10⁵ cells/dish, and maintained in Eagle’s minimal essential medium supplemented with 5% heat-inactivated calf-serum. Actinomycin D (Merck, Sharp and Dohme) was dissolved in growth medium and added to cells at a density of 3 x 10⁴ cells/cm². M-MSV was added under a vol. of 0.5 ml/tube (0.5 focus forming units (f.f.u.)/cell) or 2 ml per dish (0.1 f.f.u./cell) and adsorbed for 1 h. In all experiments cells were pre-treated for 30 min with 25 μg/ml DEAE-dextran (Duc Nguyen, 1968), and washed three times with Hanks’s balanced salt solution following virus adsorption and exposure to actinomycin D.

The infective virus produced was assayed by a simplified technique described by Guillemain et al. (1971). Undiluted culture supernatant fluids were plated on to sensitive fresh 8828 cells. The presence of infective virus in these supernatant fluids was ascertained by overall conversion of the monolayer of test-cells. It had been determined previously that overall conversion was detectable when 2 x 10⁵ f.f.u./ml of M-MSV was added.

The presence of virus particles of density 1.17 g/ml, in the supernatant fluids of infected cultures was assayed by a slight modification of the method of Robinson (1967). Cells prepared in Petri dishes were infected by M-MSV and 48 h after infection the cells were incubated in fresh medium containing 10 μCi/ml [³H]-uridine (sp. act. 20 Ci/m-mol) for 24 h. Cell culture fluids were then harvested, centrifuged at 10000 g for 20 min and layered over a 22 ml linear gradient of 20 to 70% sucrose in 10⁻³ M-tris-HCl, pH 7.2. Sedimentation was for 17 h at 24000 rev/min and 4 °C in a SW25-2 rotor.
Table 1. Effects on 8828 cells of actinomycin D at 0.25 μg/ml added for 6 h after infection by the Moloney strain of murine sarcoma virus. Assay by production of infective virus and infective centres and release of [3H]-labelled virus particles.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Untreated control cells</th>
<th>Time (h) after infection at which treatment of cells with actinomycin D commenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production of infective virus*</td>
<td>+</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>Production of [3H]-labelled virus particles (as in Fig. 1)</td>
<td>+</td>
<td>. . . .</td>
</tr>
<tr>
<td>Number of infective centres†</td>
<td>298, 259, 280 (100%)</td>
<td>33, 19, 24 (9%) 37, 20, 28 (10%) 185, 159, 158 (60%) 192, 173, 177 (65%)</td>
</tr>
</tbody>
</table>

* Undiluted supernatant fluids of infected cell cultures exposed to actinomycin D were plated on to fresh 8828 cells. The presence of infective virus in these supernatant fluids was indicated by overall conversion (+) of the monolayer of test-cells: results found in three independent experiments.

† Cells were trypsinized 24 h after infection and plated on to fresh monolayers of 8828 cells with 2 x 10⁴ infected cells per plate and 4 plates per sample. Results are the average number of foci per plate for three independent experiments.

The number of infective centres was assayed by plating cells 24 h after M-MSV inoculation (0.1 f.f.u./cell) on to fresh monolayers of 8828 cells previously seeded at a concentration of 3 x 10⁵ cells/dish. About 2 x 10⁴ infected cells were plated per dish and four dishes were used per sample. After incubation for 5 days at 37 °C, the total numbers of converted foci formed in four dishes were counted using an inverted microscope.

In a first set of experiments the actinomycin D concentration which inhibited production of infective virus was determined. Cell cultures were treated for 6 h before infection by various concentrations of actinomycin D ranging from 0.01 to 0.25 μg/ml. At 72 h after infection the cell culture fluids were assayed for infective virus. The results obtained indicated that the pre-treatment of cells with at least 0.25 μg/ml or more of actinomycin D inhibited the production of infective virus. The minimal inhibiting concentration thus determined was used in subsequent kinetic studies. For this investigation, cell cultures previously infected with M-MSV were exposed to actinomycin D at 0.25 μg/ml for 6 h at various intervals after infection and the production of infective virus, the production of virus particles of 1.17 g/ml density and the content of infective centres were assayed.

As indicated in Table 1, the production of infective virus was inhibited by actinomycin D provided cells were exposed not later than 2½ h after infection.

The presence of [3H]-labelled virus particles in supernatant fluids of infected cell cultures exposed to actinomycin D either 2 h or 4 h after infection was examined by centrifuging in 70 to 20% sucrose gradients. Fig. 1 shows that a peak of [3H]-labelled material was obtained when cells were exposed 4 h after infection. This peak disappeared when cells were actinomycin D treated as soon as 2 h after infection. This result, together with that reported above, indicates that early actinomycin D treatment inhibits the release by infected cells of both MSV and MLV particles.

Cell cultures infected with M-MSV and exposed to actinomycin D from 1 to 4 h later were then assayed for infective centres. Table 1 shows, as a percentage of the control without actinomycin D, the number of foci obtained as a function of the time between M-MSV infection and the beginning of treatment. A 90% reduction of infective centres was observed when actinomycin D was added during the 2 h following infection.
Fig. 1. Effects of actinomycin D at 0.25 µg/ml for 6 h on the release of [³H]-labelled virus particles from infected 8828 cell cultures. M-MSV infected cultures were labelled with [³H]-uridine (10 µCi/ml for 24 h). Culture supernatant fluids were then clarified and centrifuged in a 20 to 70% sucrose gradient for 17 h at 24 000 rev/min in a Spinco SW25-2 rotor. (a), control without actinomycin D; (b), actinomycin D added 2 h after infection; (c), actinomycin D added 4 h after infection.

Thus early steps in M-MSV replication *in vitro* were inhibited by treatment for 6 h with actinomycin D. The early actinomycin D sensitive period lasted for 2½ h after infection. Furthermore, the inhibition of cell conversion occurred simultaneously with the inhibition of virus production, since cells were not converted if exposed to actinomycin D within 2½ h of infection.

The mechanism of action of actinomycin D is still unclear: the cell division required for virus production may be prevented. However, Murray & Temin (1970) showed that non-dividing cells could be infected successfully with MSV, and that virus was produced when cell multiplication was resumed. In our observations a treatment of cells for 6 h with 0.25 µg/ml actinomycin D reversibly inhibited cell growth for a period no longer than 48 h, after which cells resumed normal multiplication (unpublished results). Thus the preceding hypothesis seems to be inappropriate. The 90% reduction of infective centres observed when actinomycin D was added within 2 h after infection suggests that the early actinomycin D-sensitive step of the virus replication cycle may be the synthesis of DNA-provirus. This is in accordance with the finding by Bader (1972) that the synthesis of DNA-provirus is initiated within 1 h of infection, and with the finding by McDonnell *et al.* (1970) that the *in vitro* formation of double-stranded DNA by virus-associated DNA-polymerase(s) is inhibited by this antibiotic. Actinomycin D may also act by preventing transcription of DNA-provirus.
In this case sufficient virus RNA must be synthesized within 2½ h of infection for M-MSV to be produced.

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**REFERENCES**


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