The Role of DNA Synthesis in Virus Replication and the Morphological Transformation of Normal Mouse Embryo Cells by MSV (MOLONEY)

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SUMMARY

Morphological transformation of normal mouse embryo cells by murine sarcoma virus (MOLONEY) and subsequent development of murine sarcoma virus growth were investigated using cytosine arabinoside (ara-C) and X-irradiation. Inhibition of DNA synthesis, cell division and transformation by ara-C were all reversible by deoxycytidiné. The susceptibility of mouse embryo cells to infection and morphological transformation was most sensitive to ara-C during the first 6 hr after the virus-cell encounter. Sensitivity decreased with time, and by 24 to 48 hr virtually all infected cells had become transformed and completed the virus growth cycle, despite ara-C treatment. Irradiation of transformed, virus-producing cells with either 5000 R or 100,000 R had little effect on the ability of infected cells to produce murine sarcoma virus 24 hr later, and about 10% irradiated cells were still producing virus after 48 hr. Radiation survival and heat (37°C) inactivation data for murine sarcoma virus (MOLONEY) are presented. The results indicate that the successful infection and morphological transformation of normal mouse embryo cells by murine sarcoma virus (MOLONEY) requires a DNA synthetic event immediately after the virus-cell encounter and, that once initiated, successful virus growth no longer depends on DNA synthesis.

INTRODUCTION

Infection, transformation and initiation of the growth cycle of Rous sarcoma virus (RSV) in chick embryo cells has been shown to depend on DNA synthesis in the early part of the virus growth cycle (Bader, 1964, 1965, 1966a, b; Nakata & Bader, 1968a; Temin, 1964). Although later events seem to depend on functional DNA, they do not involve con-

continued DNA synthesis. Thus actinomycin D, which inhibits DNA-dependent RNA polymerase, also inhibits RSV production (Bather, 1963; Temin, 1963; Bader, 1964). Drugs (Bather, 1963; Bader, 1966a; Temin, 1967; Nakata & Bader, 1968a), and X-irradiation (Rubin & Temin, 1959; Bader, 1966b) applied late in the cycle of RSV growth (after ‘fixation’—Nakata & Bader, 1968a) have little or no effect on virus growth and production in infected cells.

Murine sarcoma virus (MSV) (Moloney, 1966) also seems to require an early DNA synthetic event for successful infection and transformation of rat cells (Nakata & Bader, 1968b), 3T3 mouse cells (Hirschman et al. 1969) and mouse embryo cells (Buck & Bather,
1969). This requirement is probably transient and lasts for approximately 12 hr after infec-
tion of rat cells and 16 hr after infection of 3T3 cells.

The results presented here confirm and extend the latter observations to virus-cell inter-
action between MSV (MOLONEY) and normal mouse embryo cells, utilizing cytosine arabinos-
ide, a specific suppressor of DNA synthesis in chick cells (Bader, 1965), rat cells (Nakata
& Bader, 1968b) and 3T3 cells (Hirschman et al. 1969) and X-irradiation.

METHODS

Virus. Murine sarcoma virus (MSV, MOLONEY) was obtained in 1966 from Dr J. B. Moloney,
National Cancer Institute, Bethesda, Maryland, as a lyophilized tissue homogenate design-
ated ‘Rapid passage no. 26’. It has since been maintained in tissue culture and serial
amo passage in this laboratory.

Chemicals. Cytosine-β-D-arabinofuranoside (ara-C) was purchased from Calbiochem,
Los Angeles, California, and deoxycytidine hydrochloride (dC) from Mann Research
Laboratories, New York, N.Y. [3H]thymidine (sp. act. 6-7 C/mm) and [3H]uridine (sp. act.
> 2C/mm) were both obtained from New England Nuclear, Boston, Massachusetts. These
compounds were dissolved in appropriate amounts in growth medium (see below).

Tissue culture. All cells were used as secondary cultures of fibroblasts derived from primary
cultures of 15-day Swiss mouse embryo cells. These mice are random-bred in a barrier-
sustained colony maintained at this University. Growth medium consisted of powdered
medium 199 (Grand Island Biological Co., Grand Island, N.Y.) + 5% calf serum + 100
u./ml penicillin and 100 µg./ml. streptomycin. Plastic Petri dishes (Falcon Plastics, Los
Angeles, California) were used throughout. Assays of virus and transformed cells were
as previously described (Bather, Leonard & Yang, 1968). DNA and RNA synthesis were
studied by measuring the uptake of [3H]-labelled nucleotides into actively growing cells on
glass coverslips. After allowing the cells to take up the radioactive nucleotide for 1 hr the
coverslips were washed rapidly in Hanks's balanced salt solution and fixed in methanol.
Soluble nucleotides were removed by treating the coverslips in 5% trichloracetic acid for
5 min. at 4°C and washing 10 times in distilled water. After drying, incorporated radioactivity
was counted directly on the coverslips in a windowless Philips gas flow counter by the
method of Baltimore & Franklin (1962) as described previously (Buck & Bather, 1969).

X-irradiation. Irradiation was carried out with a Machlett OEG-60 tube operated at
50 kv and 20 ma. For low intensity radiation of cells the covered plastic Petri dish was
exposed at 21 cm. from the target and received a dose of 68R per sec. For high intensity
radiation the virus or cells were exposed at 1 cm. from the target and received a dose of
approximately 350R per sec. When cells were irradiated the bottom of the Petri dish was
divided into thirds and approximately one-third of the irradiated cells scraped off into 1 ml.
growth medium with a rubber policeman immediately after irradiation. These cells were
dispersed by pipetting and counted, and 1000 were plated on indicator plates of 24-hr-old
mouse embryo cells. The remaining two-thirds of cells were fed and another third scraped
off and assayed at 24 hr. The final one-third was assayed at 48 hr.

RESULTS

Effect of cytosine arabinoside on DNA and RNA synthesis

Ara-C is an effective suppressor of [3H]thymidine incorporation into the DNA of chick
cells, rat cells and 3T3 mouse cells and the effect is reversible by dC (Bader, 1965; Hirschman
DNA synthesis in MSV transformed cells

e et al. 1969; Nakata & Bader, 1968b). The effect of ara-C on incorporation of both [³H]-thymidine and [³H]uridine into normal secondary mouse embryo cells was tested by treating the cells for 6 hr with different concentrations of ara-C in growth medium immediately before feeding with the tritium-labelled precursor (Table 1) for 1 hr. It is apparent that concentrations of 10⁻³ and 10⁻⁴ M ara-C exerted approximately tenfold greater inhibition of DNA synthesis than of RNA synthesis. As shown by Nakata & Bader (1968b), the decrease in RNA activity at concentrations greater than 10⁻⁵ M ara-C can be attributed to the decrease in cell division attendant on suppression of DNA synthesis.

To study the ability of the cells to recover after ara-C treatment, they were treated with 10⁻³ M ara-C for 6 hr, then washed and fed either with fresh medium or with fresh medium containing 10⁻² M dC. At intervals after ara-C treatment, DNA synthetic activity was

Table 1. Effect of 6 hr treatment of mouse embryo cells with ara-C on their ability to synthesize DNA and RNA

<table>
<thead>
<tr>
<th>Molarity ara-C</th>
<th>[³H]thymidine uptake (%)</th>
<th>[³H]uridine uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0, 100.0</td>
<td>100.0, 100.0</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>92.5, 97.6</td>
<td>90.4, 96.0</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>60.8, 74.0</td>
<td>90.4, 93.6</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>10.1, 8.2</td>
<td>86.1, 88.5</td>
</tr>
<tr>
<td>10⁻³</td>
<td>6.0, 3.9</td>
<td>58.0, 67.9</td>
</tr>
</tbody>
</table>

Fig. 1. Recovery of the ability of mouse embryo cells to incorporate [³H]thymidine into DNA after treatment with 10⁻³ M ara-C for 6 hr. ○——○, controls; ●—●, ara-C medium replaced with growth medium + 10⁻² M dC; △—△, ara-C medium replaced with growth medium only.
measured by feeding the cells with [³H]thymidine for 1 hr. It was found (Fig. 1a, b) that, although recovery of DNA synthesis occurred in the presence or absence of dC, recovery was much more efficient in the presence of the nucleoside. Thus, as in other cellular systems, ara-C is an effective DNA synthetic inhibitor in secondary mouse embryo cells and its action is reversible.

Cell growth

To test further the action of ara-C on mouse embryo cells, 10⁶ cells were planted in 60 mm. plastic dishes. After 24 hr incubation the cells were treated for 6 hr with different amounts of ara-C in growth medium. This was replaced with fresh growth medium either with or without 10⁻² M dC and the cells counted 72 hr later. Control plates showed an approximate doubling of cell numbers between 24 and 72 hr. All levels of ara-C tested exerted inhibitory effects on cell growth (Table 2). However, the marked inhibitory action of 10⁻³ M ara-C was almost entirely counteracted by the addition of 10⁻² M dC to the medium after ara-C treatment.

<table>
<thead>
<tr>
<th>Molarity ara-C</th>
<th>Cell growth as percentage of untreated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0, 100.0</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>87.6, 60.5</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>63.0, 50.0</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>53.0, 47.0</td>
</tr>
<tr>
<td>10⁻³</td>
<td>36.9, 33.6</td>
</tr>
<tr>
<td>10⁻³+10⁻² dC</td>
<td>83.0, 79.8</td>
</tr>
</tbody>
</table>

Table 3. Effect of 6 hr pre-treatment of mouse embryo cells with ara-C on their ability to transform with MSV

<table>
<thead>
<tr>
<th>Molarity ara-C</th>
<th>Focus formation (transformation) as a percentage of untreated controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0, 100.0, 100.0</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>97.0, 71.6, 82.9</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>65.6, 57.6, 76.7</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>25.5, 34.3, 40.9</td>
</tr>
<tr>
<td>10⁻³</td>
<td>9.6, 13.8, 15.5</td>
</tr>
<tr>
<td>10⁻³+10⁻² dC</td>
<td>--, 84.8, 79.0</td>
</tr>
</tbody>
</table>

* Numbers of foci on control plates ranged between 100 and 300 per plate. All results based on duplicate counts.

Effect of ara-C on transformation of mouse embryo cells

Assay indicator plates of mouse embryo cells prepared as previously described (Bather et al. 1968) were treated for 6 hr with various concentrations of ara-C before infecting with MSV. The virus was left on the cells and fresh growth medium added to the plates. The foci developing were counted at 5 days after infection and the results expressed as percentages of control plates receiving no ara-C treatment (Table 3). It is apparent that pretreatment for 6 hr with 10⁻³ M ara-C markedly reduced the extent of transformation with MSV and that a tenfold excess of dC was sufficient to restore the transformation response almost to the level of the untreated cells.
DNA synthesis in MSV transformed cells

Effect of ara-C during development of MSV growth cycle

Table 4 shows the results of three experiments in which assay indicator cultures of mouse embryo cells were treated with $10^{-3}$ M ara-C for 6 hr at different times after infection with a standard inoculum of MSV. The virus was allowed to adsorb for 1 hr before washing off and adding growth medium with ara-C. After ara-C treatment the plates were washed and fed with growth medium containing $10^{-2}$ M dC. Transformed foci were counted 5 to 6 days later. Because of the short time of treatment with ara-C (6 hr), the long and variable latent

Table 4. Effect of 6 hr treatment with $10^{-3}$ M Ara-C on the initiation and development of foci of MSV transformed cells

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>20.9</td>
<td>25.1</td>
<td>57.3</td>
<td>98.5</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
<td>11.3</td>
<td>36.7</td>
<td>77.2</td>
<td>95.0</td>
</tr>
<tr>
<td>3</td>
<td>2.9</td>
<td>4.1</td>
<td>22.4</td>
<td>74.8</td>
<td>94.3</td>
</tr>
</tbody>
</table>

* At the end of the treatment the cells were washed twice with Hank's balanced salt solution and fed with growth medium containing $10^{-3}$ M dC.

Table 5. Effect of X-irradiation on the ability of transformed cells to produce virus

<table>
<thead>
<tr>
<th>Hours after X-irradiation</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000 R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>406</td>
<td>358</td>
<td>221</td>
</tr>
<tr>
<td>24</td>
<td>223</td>
<td>238</td>
<td>161</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>104</td>
<td>144</td>
</tr>
<tr>
<td>10^5 R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>398</td>
<td>250</td>
<td>191</td>
</tr>
<tr>
<td>24</td>
<td>228</td>
<td>125</td>
<td>144</td>
</tr>
<tr>
<td>48</td>
<td>146</td>
<td>55</td>
<td>20</td>
</tr>
</tbody>
</table>

* Each number represents the mean of two plates.

period of the MSV growth cycle and the rapid recovery of cells in the presence of dC, complete inhibition of foci formation was not seen in these experiments. However, it is clear that ara-C is most effective within 6 hr of infection. If treatment starts 12 hr after infection, 20 to 40% of the infected cells complete the cycle and produce virus which results in the development of foci. At 24 hr about 75% of the infected cells complete the growth cycle and at 48 hr almost 100% do so. These results indicate a variable eclipse phase which may extend in some cells to beyond 24 hr and emphasize that an early DNA synthetic event is necessary for virus growth and cell transformation.
Effect of X-irradiation on ability of transformed cells to produce virus

It was previously shown (Bather et al. 1968) that inhibition of cell division by 5000 R X-irradiation of indicator cells completely prevented the formation of foci by MSV. However, no experiments have been reported to show whether MSV infected cells continue to produce virus after irradiation. As a preliminary, the rate of inactivation of MSV by X-irradiation and the half life of MSV in culture medium at 37 ° were determined. From three experiments in which MSV was irradiated at an intensity of approximately 20,000 R/min., the decay constant was calculated to be 0·124 ± 0·008 min.−1 and was similar to that for RSV and Newcastle disease virus (Rubin & Temin, 1959). This supports a similarity of structure and type of nucleic acid for these viruses; probably single-stranded RNA of molecular weight approximately 10^7 (Robinson, Robinson & Duesberg, 1967). The rate of heat inactivation of MSV at 37 ° in growth medium indicated a half life of 5·1 hr.

When transformed MSV-producing cells were X-irradiated at 2 dose levels (5000 R and 100,000 R) and then assayed on mouse embryo indicator cells for virus producing capacity 24 hr later, it was found that the irradiated cells produced virus with almost the efficiency of untreated cells (Table 5). More than 10 % of the cells were producing virus 48 hr after irradiation and cessation of DNA synthesis and cell growth.

DISCUSSION

The results reported here on the effect of ara-C and X-irradiation on the ability of normal mouse embryo cells to become transformed by MSV and support its growth, confirm and extend those reported previously for rat and 3T3 mouse cells (Nakata & Bader, 1968b; Hirschman et al. 1969). They also extend the observations that many properties of the mouse sarcoma-leukaemia group of viruses are very similar to those of the avian sarcoma-leukaemia group.

Since it has been shown that requirements for infection and transformation by MSV include the ability of the cells to divide and undergo DNA synthesis (Hartley & Rowe, 1966; Bather et al. 1968; Nakata & Bader, 1968b; Buck & Bather, 1969; Hirschman et al. 1969) it was necessary to determine the action of ara-C on both cell growth and DNA synthesis. The experiments reported here show that cell division was sensitive to a 6-hr treatment with ara-C but that this could be reversed by the addition of deoxycytidine. Similarly, DNA synthesis was highly sensitive to ara-C but could also be reversed by an excess of dC. RNA synthesis was much less affected by ara-C, and the decrease in RNA activity with high doses of ara-C could be attributed to lowered cellular activity attendant upon decreased cell division.

When the effect of ara-C on focus production (infection and transformation) was studied, it was apparent that inhibition of focus production followed very closely the inhibition of DNA synthesis and cell division and that dC reversed all three very effectively. Thus, the transformation of normal mouse embryo cells by MSV is dependent on DNA synthesis, as is the transformation of 3T3 cells (Hirschman et al. 1969) and of rat cells (Nakata & Bader, 1968b). The kinetics of virus production (Table 4) show that infected mouse cells are sensitive to ara-C inhibition of MSV production immediately after infection and that this sensitivity gradually disappears until at 48 hr after infection the cells produce virus as efficiently as untreated controls. No complete inhibition was observed since treatment with ara-C was carried out in pulses of 6 hr duration beginning at different times after infection. Hirschman et al. (1969) reported complete inhibition of MSV growth in 3T3 cells if
ara-C was applied continuously for 16 hr after infection. However, MSV adsorption to mouse embryo cells is slow and variable (Bather et al. 1968) and the cells rapidly recover their ability to synthesize DNA after ara-C treatment for 6 hr if dC is present (Fig. 1). It is to be expected, therefore, that a small proportion of affected cells will recover sufficiently to complete and continue the infectious cycle.

The sensitivity of MSV (MOLONEY) to X-irradiation is very similar to that of RSV and Newcastle disease virus (Rubin & Temin, 1959) and is such that an exposure of 5000R has little effect on infectivity. An exposure of 100,000R, on the other hand, reduces the infectivity of free virus by about 25%. Both doses would certainly inhibit all cellular DNA synthesis and cell division and it has been shown that irradiation with 5000R completely inhibits the transformation of mouse embryo cells by MSV (Bather et al. 1968). Exposure of already infected, virus-producing cells to low or high doses of X-irradiation had very little effect on their ability to produce virus 24 hr later. Even after 48 hr, more than 10% of the irradiated cells were still producing virus. That this is not residual virus released from inactivated cells is clear from the results of MSV survival at 37°. With a half life of approximately 5 hr, only about 3% of the extracellular virus would survive for 24 hr and less than 0.2% would survive for 48 hr. Since MSV is highly susceptible to damage by the procedures of freezing and thawing or sonication used to break open cells (unpublished results), it was not feasible to compare the infectivities of unbroken and broken irradiated cells. However, it seems unlikely that the infectivity of the cells 48 hr after irradiation was due to residual cell attached virus in view of the indication that the infectivity present was more than 10 times that expected if residual virus was responsible.

Many reports have indicated that most mouse embryo cells morphologically transformed by MSV (MOLONEY) in vitro cannot grow progressively to form foci as can chicken cells transformed by RSV (Hartley & Rowe, 1966; O’Connor, 1968; Yoshikura et al. 1968; Bather et al. 1968). Focus formation seems to depend primarily on secondary infection followed by morphological transformation of the infected cells. However, mouse and hamster cells transformed by MSV (HARVEY) as well as rat and mouse Balb 3T3 cells transformed by MSV (MOLONEY) can grow continuously in tissue culture (Simons et al. 1967a, b; Ting, 1966; Todaro & Aaronson, 1969). The reason for the failure of mouse embryo cells to grow and multiply after MSV (MOLONEY) infection is not known and may be a peculiarity of the system (strain of mouse, coproduction of large numbers of murine leukaemia virions, etc.). In our hands, MSV (MOLONEY) induces tumours in almost 100% of newborn mice of the strain used for preparation of the tissue cultures. However, in view of the growth reluctance of the in vitro transformed cells, the conclusion from these experiments is that replication of MSV, which is required for secondary infection and the predegenerative morphological transformation, requires an early DNA synthetic event which is inhibited by cytosine arabinoside. The duration of this requirement is variable but begins immediately after infection and may continue for 24 hr in some cells. By 48 hr after infection, DNA synthesis and cell division are no longer necessary for virus growth. Thus, in these respects, the virus-cell interaction between MSV and mouse cells resembles that between RSV and chick cells (Bader, 1964, 1965, 1966a, b; Temin, 1964; Nakata & Bader, 1968a) and indicates a requirement for DNA synthesis for fixation of the viral genome in mouse embryo cells.
REFERENCES


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