The Inhibition of Polyoma Antigen Synthesis and Capsid Formation by 5-fluorodeoxyuridine

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(Accepted 24 February 1967)

SUMMARY

In mouse embryo cells infected with polyoma virus, inhibition of DNA replication by fluorodeoxyuridine (10 μM) suppressed the production of haemagglutinin, infectious virus and viral antigen as judged by direct immunofluorescence. No virus-like particles were detected in extracts of inhibited cultures examined by electron microscopy.

INTRODUCTION

Inhibitors of functional DNA synthesis have been extensively used to study the outcome of viral infection in terms of appearance of cytopathic effects, production of antigenic material and of actual virus-like particles. Some of the metabolic inhibitors, like the halogenated bases bromo- and iododeoxyuridine, or the antibiotic mitomycin C, allow a limited synthesis of DNA strands, which, however, are non-functional because of base substitution or cross-linking; others, like fluorodeoxyuridine, prevent DNA synthesis by interfering with thymidylate production (Cohen et al. 1958).

The reported finding of polyoma particle production in mouse embryo cultures inhibited with fluorodeoxyuridine (Sheinin, 1964) seemed to warrant a more detailed study of such particles and of their structural and morphological characteristics; all attempts to produce them under the conditions used in this laboratory were, however, unsuccessful. This paper presents evidence that inhibition of DNA replication with fluorodeoxyuridine in mouse embryo cells infected with polyoma virus prevents the production of virus protein and capsid assembly.

METHODS

Viruses. The derivation and properties of the large- and small-plaque lines of polyoma virus used were described by Diamond & Crawford (1964); polyoma virus was used either as crude high-titre stock preparations (Crawford, 1962), or after purification by equilibrium centrifugation in caesium chloride density gradients (Crawford, Crawford & Watson, 1962).

Media and solutions. ETC10 was Eagle's medium modified to contain twice the standard concentration of amino acids and vitamins, plus 10 % (v/v) tryptose phosphate broth (Difco) and 10 % (v/v) calf serum. ETH had 2-5 % (v/v) horse serum instead of calf serum. EDCS was modified Eagle's medium with 5 % (v/v) calf serum dialysed against phosphate buffered saline (Dulbecco & Vogt, 1954). 2-amino-2-
hydroxymethylpropane-1,3-diol (tris) saline was the TD of Smith et al. (1960).

Medium IO66 was as described by Parker, Castor & McCulloch (1957), minus thymidine, minus coenzymes.

Cells. Secondary mouse embryo cells were grown in 50 × 10 mm. plastic Petri dishes or in 80 oz. roller bottles (House & Wildy, 1965) in ETC in equilibrium with 5 % (v/v) CO₂ in air.

Chemicals. The 5-fluorodeoxyuridine (FUDR) was kindly given by Hoffmann-La Roche Inc., Nutley, N. J. Uridine, A grade, and thymidine, A grade, were purchased from CalBioChem, 3625 Medford Street, Los Angeles, California, U.S.A. Stock solutions (10 mM) were made in tris saline, filtered through Millipore 0.45 µ membranes and stored at -20°.

Experimental procedure. Cultures were infected, before they became confluent, at input multiplicities varying from 15 to 1700 plaque forming units (p.f.u.)/cell; inoculum volume was 0.2 ml. for Petri dishes and 10 ml. for bottle cultures; adsorption was at 37°, in a CO₂ incubator, for 1 hr. Cultures were then washed 3 times with warm tris saline, and incubated with EDCS with the appropriate concentration of drugs. Assays for background virus were made during the latent period; assays for virus yields were made at 32 to 48 hr after infection.

Receptor destroying enzyme (RDE) was prepared from Vibrio cholerae filtrate and tested according to Burnet, McCrea & Stone (1946); its activity was 512 units/ml.

Extraction of the virus. Cells were scraped into the medium, cooled to 4° to allow virus adsorption, centrifuged, and resuspended in tris saline (pH 8.5, 0.2 ml. per dish). The cells were disrupted by ultrasonic treatment and the cell debris removed by centrifugation (700 g for 15 min.). The supernatant fluid was the 1st tris extract; the pellet of cell debris was resuspended in tris saline with 20 % (v/v) crude RDE and incubated at 37° for 24 hr; the suspension was clarified by centrifugation (1st RDE extract). The procedure was repeated until the haemagglutinating titres of the extracts were less than 1/10. Infectivity assays were made on the extracts with the maximum haemagglutinating activity. In the experiments with the large-plaque strain the cell suspension was acidified by adding phosphate buffer (0.4 M, pH 5.6); after cooling to 4°, cells were centrifuged and suspended in tris saline (pH 8.5), frozen and thawed 3 times, incubated 30 min. at 45°, and cleared by centrifugation. This gentler method was relatively ineffective with the small-plaque virus, but was used to prepare extracts for electron microscopy, starting from large bottle cultures.

Infectivity assays. Plaque assays for polyoma virus were made as described by Crawford et al. (1962).

Haemagglutinin assays were done in Perspex trays, using phosphate-buffered saline (a) as diluent, with 0.2 ml. virus dilutions and 0.2 ml. 1 % (v/v) guinea-pig red blood cells; haemagglutination patterns were read after complete sedimentation at 4°. The amount of virus present in the last cup with complete haemagglutination was considered the haemagglutinating unit (HAU).

Electron microscopy. Particle counts were made by the loop-drop method of Watson, Russell & Wildy (1963); equal amounts of virus sample, latex emulsion (Dowex, 1880 Å diameter, 1.2 × 10⁹ particles/ml.) and potassium phosphotungstate (2 % w/v, pH 7.2) were mixed in a small vial; drops of the mixture were transferred with a platinum loop to carbon-coated grids, and excess fluid was drained by touching lightly with filter paper. The preparations were observed in a Siemens Elmiskop I at a magni-
Inhibition of polyoma virus synthesis by FUdR

Inactivation of x 40,000. RDE extracts were usually unsatisfactory for accurate counts because of stain precipitation; tris extracts gave much cleaner and better contrasted preparations. The most reliable counts were made on concentrated and purified yields from large cultures.

Immunofluorescence. Rabbits were hyperimmunized with purified polyoma antigen (empty particles) and γ-globulin was conjugated with fluorescein isothiocyanate as described by Fraser & Crawford (1965).

RESULTS

Inhibition of infectious virus yields

The production of infectious virus was completely inhibited by FUdR (10 μM) at all multiplicities of infection tested. The infectious yields of cultures infected at a multiplicity of infection of about 100 p.f.u./cell decreased with increasing concentrations of FUdR (Table 1). At higher multiplicities of infection control yields showed only a small increase over background levels, and FUdR inhibition was correspondingly small. The FUdR inhibition of infectious yields was partially reversed by the presence of equimolar thymidine (Table 2).

Table 1. Depression of infectivity and haemagglutinating activity in polyoma infected cultures incubated with different amounts of FUdR

<table>
<thead>
<tr>
<th>Concentration of FUdR (μM)</th>
<th>p.f.u. 44 hr</th>
<th>HAU 44 hr</th>
<th>p.f.u. 0 hr</th>
<th>HAU 0 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.1 × 10⁵</td>
<td>1.9 × 10⁵</td>
<td>400</td>
<td>16,000</td>
</tr>
<tr>
<td>0.01</td>
<td>4.9 × 10⁵</td>
<td>2.8 × 10⁵</td>
<td>400</td>
<td>1,600</td>
</tr>
<tr>
<td>0.1</td>
<td>4.4 × 10⁵</td>
<td>1.4 × 10⁵</td>
<td>320</td>
<td>820</td>
</tr>
<tr>
<td>1</td>
<td>5.3 × 10⁵</td>
<td>7.5 × 10⁴</td>
<td>320</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>4.4 × 10⁵</td>
<td>4.2 × 10⁴</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>100</td>
<td>5.1 × 10⁵</td>
<td>5.5 × 10⁴</td>
<td>400</td>
<td>200</td>
</tr>
</tbody>
</table>

The titre of infectious virus and haemagglutinating virus was determined at the beginning of the experiment and at 44 hr. The inoculum was purified small-plaque polyoma virus; input multiplicity of infection was 100 p.f.u./cell. The incubation medium was EDCS with uridine (10 μM) and the indicated concentration of FUdR.

Table 2. The effect of thymidine and FUdR on the infectious and haemagglutinating yields of polyoma infected cultures

<table>
<thead>
<tr>
<th>Concentration of thymidine (μM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of FUdR (μM)</td>
<td>p.f.u.</td>
<td>HAU</td>
<td>p.f.u.</td>
</tr>
<tr>
<td>0</td>
<td>4.0 × 10⁶</td>
<td>5120</td>
<td>7.6 × 10⁵</td>
</tr>
<tr>
<td>0.1</td>
<td>1.5 × 10⁷</td>
<td>80</td>
<td>1.2 × 10⁷</td>
</tr>
<tr>
<td>1</td>
<td>6.5 × 10⁴</td>
<td>≤ 20</td>
<td>7.7 × 10⁴</td>
</tr>
<tr>
<td>10</td>
<td>8.3 × 10⁴</td>
<td>≤ 20</td>
<td>7.9 × 10⁴</td>
</tr>
<tr>
<td>100</td>
<td>8.1 × 10⁴</td>
<td>≤ 20</td>
<td>3.2 × 10⁷</td>
</tr>
</tbody>
</table>

Inoculum was small-plaque polyoma virus; input multiplicity of infection was 20 p.f.u./cell. Yields were assayed 40 hr after infection; background activity was 5 to 10 × 10⁴ p.f.u., ≤ 20 HAU. Low titres of haemagglutinating virus could not be estimated owing to the presence of RDE in the extracts.
Inhibition of haemagglutinin yields

The haemagglutinating activity of virus yields should not be affected if FUdR specifically inhibits DNA replication without impairing viral protein synthesis and capsid assembly (Sheinin, 1964). This was shown not to be the case, since in all experiments FUdR (10 μM) prevented the rise of haemagglutinating activity over background levels; lower concentrations of the drug reduced the yield of haemagglutinin (Table 1), and the thymidine reversal of FUdR inhibition of infectivity was accompanied by restoration of haemagglutinin production (Table 2). The relative insensitivity of haemagglutinin assays made them unsuitable for studying the effect of FUdR at low multiplicities of infection.

Table 3. FUdR depression of particle production in polyoma infected mouse embryo cells

<table>
<thead>
<tr>
<th>Expt A*</th>
<th>Control</th>
<th>10 μM FUdR</th>
<th>1 hr (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles</td>
<td>2.4 x 10^11</td>
<td>&lt; 5 x 10^7</td>
<td>&lt; 5 x 10^7</td>
</tr>
<tr>
<td>p.f.u.</td>
<td>1.6 x 10^9</td>
<td>3.2 x 10^8</td>
<td>3.6 x 10^8</td>
</tr>
<tr>
<td>HAU</td>
<td>80,000</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt B†</th>
<th>Control</th>
<th>10 μM FUdR</th>
<th>1 hr (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles</td>
<td>1.9 x 10^11</td>
<td>&lt; 5 x 10^7</td>
<td>&lt; 5 x 10^7</td>
</tr>
<tr>
<td>p.f.u.</td>
<td>8.1 x 10^8</td>
<td>4.1 x 10^8</td>
<td>4.5 x 10^8</td>
</tr>
<tr>
<td>HAU</td>
<td>40,000</td>
<td>&lt; 50</td>
<td>&lt; 50</td>
</tr>
</tbody>
</table>

80 oz bottle cultures containing about 2 x 10^8 cells were infected with 3 x 10^9 p.f.u. small-plaque polyoma virus, either purified (Expt A) or crude (Expt B). Incubation medium was EDCS with uridine (10 μM).

*Yields were assayed 1 and 48 hr after infection, on freeze-thawed extracts.
†Yields were assayed 1 and 44 hr after infection, on freeze-thawed extracts.

Inhibition of particle production

The lack of haemagglutinin production in the cultures inhibited by FUdR might have been due to the lack of coat protein subunits, or to the failure of the subunits to assume the spatial arrangement required for the expression of haemagglutinating activity. To investigate the latter possibility, attempts were made to detect the production of morphological particles in cultures in which infectious virus yields were suppressed by FUdR, but while large numbers of virus particles, both ‘full’ and ‘empty’ (Crawford et al. 1962), were seen in extracts from control cultures, no comparable particles could be detected in cultures inhibited by FUdR (10 μM). More concentrated material was prepared by infecting large bottle cultures; particle counts were high from control cultures but not from FUdR cultures (Table 3).

Inhibition of viral antigen synthesis

The effect of FUdR (10 μM) on the synthesis of antigenic protein was next investigated by direct immunofluorescence. In control cultures infected at a multiplicity of 200 p.f.u./cell, 32.6 % of the nuclei showed bright fluorescence in the pattern typical of polyoma infection, and some cells showed fluorescent cytoplasmic granules; in
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cultures incubated with FUdR none of the cells showed the extensive nuclear fluorescence of positive controls, and only 3.7% showed small bright specks over the nucleus.

Effect of culture media and virus strain on FUdR inhibition

The results presented so far were obtained in experiments performed with Eagle's medium. Since previous work with the more complete culture medium 1066 had led to different results (Sheinin, 1964) we thought it important to compare the two media to see how they affected the action of FUdR on polyoma virus replication. Parallel cultures were infected with small-plaque polyoma virus at a multiplicity of infection of about 10 or 1000 p.f.u./cell, and incubated with FUdR and control media. Control media were Eagle's or 1066, both enriched with 5% (v/v) dialysed calf serum and supplemented with thymidine (41 μM). FUdR media had FUdR (10 μM) instead of thymidine. Whichever medium was used the result was the same; in both, FUdR suppressed the production of haemagglutinin and the formation of morphologically detectable particles. Comparable results were also obtained with both media using the large-plaque variant of polyoma virus.

DISCUSSION

In the present series of experiments polyoma virus multiplication in mouse embryo cells was prevented by the presence of FUdR, a known inhibitor of DNA replication. Inhibited cultures produced neither haemagglutinin nor virus-like particles, and none of their cells synthesized viral antigens identifiable by immunofluorescence. One may conclude that the infecting genetic material is not able, without replication, to direct the synthesis and assembly of detectable amounts of viral protein subunits.

Sheinin (1964) observed production of virus-like particles and antigenic material, as shown by immunofluorescence and serum blocking power, in mouse-embryo cells infected with polyoma virus and incubated in the presence of FUdR (10 μM). It is difficult to reconcile the two sets of observations, whose difference could not be accounted for by the use of different tissue culture medium in our earlier experiments. It is still possible that the experimental conditions are in some way different; if so, the conditions under which viral protein synthesis and assembly are independent of DNA replication have still to be defined.

It is a pleasure to express my gratitude to Professor M. G. P. Stoker for the hospitality of the Institute of Virology; I am also grateful to Dr L. V. Crawford for his guidance throughout this work and to the other members of the staff for helpful advice and criticism; Dr E. A. C. Follett and Miss M. Young helped with the electron microscope work. I am also indebted to Dr R. Sheinin for the constructive interest she showed in this work, and for the generous gift of her incomplete medium 1066. I was supported by a research grant from the Istituto Sieroterapico e Vaccinogeno Toscano 'A. Sclavo', Siena.
REFERENCES


(Received 14 December 1966)