The Effect of 5-fluoro-deoxyuridine on the Replication of Viral DNA and Synthesis of Polyoma Capsid Protein

(Accepted 15 November 1967)

It has been reported that synthesis of specific viral protein requires replication of viral DNA. Various investigators (Flanagan & Ginsberg, 1962; Kjellén, 1962; Gilead & Ginsberg, 1965) found that the formation of virus specific proteins in adenovirus-infected cells was directly dependent upon the synthesis of viral DNA. The analogue 5-fluoro-2-deoxyuridine (FUdR) completely inhibited synthesis of viral antigen in human foetal muscle cells infected with cytomegalovirus (Goodheart, Filbert & McAllister, 1963) and suppressed the production of specific virus antigen in SV40 infected monkey kidney cells (Melnick, Stonebaugh & Rapp, 1964; Gilden et al. 1965).

In contrast, the work on pseudorabies virus (Reissig & Kaplan, 1962) suggested that specific viral protein formation can proceed in the absence of apparent viral DNA replication and synthesis of viral protein under conditions of FUdR inhibition has been reported for vaccinia virus (Shatkin, 1963; Salzman, Shatkin & Sebring, 1963; Loh & Payne, 1965) and polyoma virus (Sheinin, 1964).

In the present work the specific polyoma capsid synthesis was inhibited when viral DNA replication was prevented by FUdR. After this manuscript was completed, a report by Urbano (1967) appeared presenting similar results.

Throughout the work, primary cultures of mouse embryo cells were used. These were made from 18- to 20-day-old embryos (Charles River Breeders, Brockton, Mass.) and five to six million cells were seeded per 60 by 15 mm. Petri dish with either Eagle's (Eagle, 1959) or Parker's 1066 medium + 10% foetal calf serum. Polyoma virus was prepared as previously described (Khare & Consigli, 1965). Mouse embryo cells were infected at a virus:cell ratio of 800:1 giving 30 to 50% infected cells. Virus was assayed by plaques and haemagglutination (Consigli, Minocha & Abo-Ahmed, 1966).

The synthesis of viral capsids was detected by immune precipitation, immunofluorescence and electron microscopy. Infected cells were washed free of unadsorbed virus after 3 hr with Hanks's solution (HBS). Five hr after infection Eagle's medium containing [3H]valine (2 μc/ml.) + 5% dialysed foetal calf serum was added, and after incubation at 37° for a total of 35 hr all cultures were harvested. Precipitable virus protein was detected by the indirect method (Shatkin, 1963). Cell extracts, prepared by freezing and thawing, were mixed with rabbit anti-polyoma capsid serum (prepared by injecting rabbits with CsCl purified, inactivated virus) and incubated for 2 hr at 37°; anti-rabbit serum (prepared in chickens) was then added and the mixture again incubated for 2 hr at 37°. The precipitates were washed four times with phosphate buffered saline, hydrolysed with NaOH and the radioactivity counted. Fluorescence microscopy was done on cells growing on coverslips using the indirect fluorescent antibody method. The percentage of cells showing nuclear fluorescence was determined on cultures incubated for 24 hr after infection; at least 200 cells were counted. Capsids were counted with a Hitachi HUB microscope using a method based on the
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The synthesis of polyoma virus and capsid protein was examined using Eagle's medium with and without 20 μM-5-FUdR which was added at different times after infection. After 35 hr cell extracts were assayed for plaques, haemagglutinin and precipitin; cells were examined for immunofluorescence after 24 hr (Table 1). Clearly, the FUdR added before and at 15 hr after infection inhibited the formation of infectious virus and structural antigens; when added at 20 hr, there was less inhibition.

Table 1. The effect of FUdR on synthesis of polyoma capsid

<table>
<thead>
<tr>
<th>Time of Addition</th>
<th>p.f.u./ml.</th>
<th>HA/ml.</th>
<th>Nuclear fluorescence (%)</th>
<th>Immuno-precipitation (counts/min./mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No FUdR harvested 10 hr</td>
<td>3·2 × 10⁴</td>
<td>64</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>No FUdR harvested 35 hr</td>
<td>1·5 × 10⁶</td>
<td>540</td>
<td>43·0</td>
<td>61·8</td>
</tr>
<tr>
<td>FUdR from 5 hr</td>
<td>3·0 × 10⁴</td>
<td>64</td>
<td>3·2</td>
<td>0</td>
</tr>
<tr>
<td>FUdR from 10 hr</td>
<td>2·2 × 10⁴</td>
<td>56</td>
<td>2·2</td>
<td>0</td>
</tr>
<tr>
<td>FUdR from 15 hr</td>
<td>2·8 × 10⁴</td>
<td>56</td>
<td>2·6</td>
<td>0</td>
</tr>
<tr>
<td>FUdR from 20 hr</td>
<td>2·2 × 10⁴</td>
<td>120</td>
<td>12·0</td>
<td>2·3</td>
</tr>
</tbody>
</table>

Table 2. Synthesis of capsids in the presence of FUdR and 5-methyldeoxycytidine

<table>
<thead>
<tr>
<th>Condition</th>
<th>p.f.u./ml.</th>
<th>HA/ml.</th>
<th>Nuclear fluorescence (%)</th>
<th>Particle counts (× 10⁻¹⁰/ml.)</th>
<th>Complete</th>
<th>Incomplete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyoma virus</td>
<td>3·0 × 10⁶</td>
<td>512</td>
<td>49</td>
<td>41</td>
<td>41</td>
<td>15</td>
</tr>
<tr>
<td>Polyoma virus + FUdR</td>
<td>3·4 × 10⁶ (1·1)</td>
<td>64 (12)</td>
<td>2 (4)</td>
<td>1·4 (3·4)</td>
<td>1·2 (8)</td>
<td>6·4 (42)</td>
</tr>
<tr>
<td>Polyoma virus + FUdR + 5MdC</td>
<td>8·0 × 10⁴ (2·6)</td>
<td>256 (50)</td>
<td>19 (38)</td>
<td>1·5 (3·7)</td>
<td>6·4 (42)</td>
<td>1·2 (8)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages of control cultures with no FUdR.

This result is in disagreement with the results of Sheinin (1964) who reported synthesis of capsid protein with apparent inhibition of polyoma DNA synthesis by FUdR. We therefore compared the ability of Eagle's and Parker's 1066 media to support the formation of capsid antigens in the presence of FUdR. Both media supported the formation of infectious virus in mouse embryo cells and FUdR was an effective inhibitor in both. But while in Eagle's medium FUdR also inhibited specific protein synthesis (using serum blocking power or immunofluorescence) it evidently failed to do so in 1066 medium. When comparing the compositions of these two media, we noticed that Parker's 1066 contained 5-methyl deoxycytidine (5MdC). This compound interested us since upon deamination it would yield thymidine and partially relieve the inhibition by FUdR. Accordingly, using Eagle's medium, we examined the ability of 5MdC to reverse inhibition by FUdR of the synthesis of viral capsid protein. In this experiment plaques, haemagglutinin and particles were assayed at 35 hr and cells were examined for immunofluorescence at 24 hr after infection (Table 2). When
infected cells were maintained in Eagle's medium containing 20 \( \mu \)M-FUdR there was synthesis of neither infective virus nor capsid proteins. When 5Mdc was included with the inhibitor there was a slight increase in infectivity and a five- to tenfold increase in polyoma capsid proteins.

Because of the above findings an experiment was done to see if 5Mdc would allow de novo viral DNA synthesis in the presence of the inhibitor. Incorporation of [\(^3\)H]-deoxycytidine into the progeny virus was investigated. Primary mouse embryo cells grown in Eagle's medium containing or lacking 5-methyl deoxycytidine were infected and unadsorbed virus was removed by washing three times 3 hr later with HBS. Eagle's medium + 5 % dialysed foetal calf serum + [\(^3\)H]deoxycytidine (1 \( \mu \)C/ml) with or without additives was then added. The additives were either 20 \( \mu \)M-FUdR or 20 \( \mu \)M-FUdR + 5Mdc (10 \( \mu \)g./ml). All cultures were harvested 35 hr after infection, extracts were concentrated in the Spinco SW 25-1 rotor at 80,000g for 3 hr. The resuspended viral pellets were treated with DNase and RNase (each 30 \( \mu \)g./ml.) for 1 hr at 37\( ^\circ \). They were then centrifuged 150,000g in the Spinco SW–39 rotor for 2 hr, removing the virus from the enzymes and degraded products in the supernatant. The virus was resuspended in a minimum (0.5 ml) of tris-buffered saline (0.01 M tris, pH 7.2; 0.15 M NaCl) and purified in a pre-set (1.20 to 1.40 g./cm\(^3\)) CsCl density gradient (Spinco SW–39 rotor) at 150,000g for 20 hr. Fractions of 12 drops each were collected from the gradient using a Model D density flow fractionometer (Instrumentation

![Graph](image)

**Fig. 1.** [\(^3\)H]deoxycytidine (1 \( \mu \)c./ml. medium) incorporation into the DNA of polyoma progeny obtained from mouse embryo cell cultures maintained in: Eagle's medium (a); Eagle's medium with FUdR (2 \times 10^{-5} \text{M}) (b); and Eagle's medium containing FUdR and 5Mdc (10 \( \mu \)g./ml. medium) (c). Cultures were harvested 35 hr after infection and purified by CsCl density gradient centrifugation. Counts/min., \( \Delta \); haemagglutinin, O; p.f.u., •.
Specialities, Lincoln, Nebraska) and assayed for plaques, haemagglutinin and radioactivity. The density gradient profiles of the virus obtained with and without added inhibitor and antagonist are interesting (Fig. 1). In Eagle's medium alone (Fig. 1a), complete virus was synthesized; determined by the incorporation of the radioisotope into the complete virus band (buoyant density 1.328 g./c.c.). In Eagle's medium + 20 μM-FUdR (Fig. 1b), the lack of incorporation observed suggested that the virus profile was composed of residual virus. However, in Eagle's medium + 20 μM-FUdR + 5MdC (10 μg./ml.) (Fig. 1c), there was 6.6% incorporation of isotope and 5% infectivity demonstrating de novo synthesis of viral DNA and indicating a partial reversal of the FUdR inhibition by 5MdC.

These findings show that 5MdC is capable of partially reversing the FUdR effect. Cells grown in the presence of 5MdC probably induce the activation of 5-methyl deoxycytidylid deaminase which deaminates 5MdC forming a limited amount of TdR. This enzyme has been previously reported in other cells (Maley & Maley, 1959, 1960; Scarano & Maggio, 1959; Keck, Mahler & Fraser, 1960) and its presence in mouse embryo cells is now being investigated in our laboratory.

The polyoma + mouse cell interaction may be analogous to that of Escherichia coli + T-even phage where it was established that an orderly sequential reading of the phage genome occurred with the production of different messenger RNAs at different times in the infectious cycle (Kano-Sueoka & Spiegelman, 1962; Sekiguchi & Cohen, 1963; Hall, Nyggard & Green, 1964). Messenger RNA made in the absence of DNA synthesis had the capacity to specify the subsequent synthesis of early, but not late, proteins. It was also found that the synthesis of early messenger stopped and that late messenger began about the time that DNA replication was initiated. This switch-off did not occur when phage DNA synthesis was prevented (Sekiguchi & Cohen, 1964). An identical mechanism may be present in the mouse cell infected with polyoma virus where the synthesis of late messenger RNA is dependent on some viral DNA replication for the synthesis of viral structural proteins.

The authors wish to thank Dr C. M. To for his time and assistance in the electron microscope studies. This investigation was supported by Public Health Service Research Grant CA 07139 from the National Cancer Institute, United States Public Health Service. R.A.C. is a recipient of a U.S.P.H. Career Development award 1-K3 CA 12056 from the National Cancer Institute. A preliminary report of this work was presented at the New York meeting of the American Society for Microbiology (1967).

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(Received 18 October 1967)