The Infectivity of Polyoma Virus DNA for Mouse Embryo Cells in the Presence of Diethylaminoethyl-dextran

By D. WARDEN* AND H. V. THORNE†

Institute of Virology, University of Glasgow, Glasgow, Scotland

(Accepted 16 May 1968)

SUMMARY

Plaques were produced in mouse-embryo monolayers by infection with polyoma virus DNA in the presence of diethylaminoethyl-dextran. Optimum conditions for plaque assay were established and dose-response relationships for component I and component (II+III) polyoma DNA determined. Efficiencies up to 6 × 10⁵ p.f.u./μg. were obtained for component I DNA.

INTRODUCTION

A plaque assay for poliovirus RNA infectivity, 20 to 100 times more efficient than hypertonic methods, in which monolayers were infected under isotonic conditions in the presence of diethylaminoethyl-dextran (DEAE-D), was described by Vaheri & Pagano (1965).

The present work shows that DEAE-D also promotes formation of plaques in monolayers of mouse embryo cells infected with polyoma virus DNA, thus providing a simple method for estimating the relative infectivities of different components of polyoma virus DNA.

METHODS

Cell culture, virus growth and purification

The methods for cell culture and growth and purification of small-plaque polyoma virus (Diamond & Crawford, 1964) were described by Thorne (1967).

Extraction of DNA

DNA was obtained from unpurified virus preparations suspended in pH 7·4 versenate buffer (Weil, 1961) containing 0·14 m-NaCl, 0·003 m-KCl, 0·001 m-KH₂PO₄, 0·008 m-Na₂HPO₄ and 0·01 m-EDTA (not 0·0005 m as used by Weil, 1961) by extraction three times with freshly distilled 80% phenol in pH 7·2, 0·02 m-phosphate buffer. Phenol was removed by ether extraction and residual ether by evaporation with a stream of nitrogen. DNA was also extracted from purified virus by heating for 10 min. at 56° in pH 8·5, 0·02 m-tris, 0·1 m-NaCl and 0·01 m-EDTA, containing 0·2 % sodium dodecyl sulphate.

Mouse DNA was extracted from mouse-embryo tissue by the procedure of Marmur (1961).

* Present address: Glaxo Laboratories, Greenford, Middlesex.
† Member of the Medical Research Council Experimental Virus Research Unit.
Sucrose-gradient fractionation of DNA

DNA from purified virus was fractionated into I and (II+III) components (Weil & Vinograd, 1963) by centrifugation on 5 to 20% sucrose gradients containing NaCl+EDTA (pH 8.5, 0.02 M-tris+HCl buffer, 0.1 M-NaCl, 0.01 M-EDTA) for 15 hr at 23,000 rev./min. in the Spinco SW 25 rotor. Fractions comprising the central regions of the I and II+III peaks were pooled and stored at 4°.

Measurement of DNA concentration

DNA concentration was estimated by measurement of u.v. absorption at 260 nm.

Plaque assay

Mouse-embryo monolayers were prepared by seeding 60 mm. Petri dishes with 2 to 3×10⁶ cells from secondary whole-mouse-embryo cultures, and were used when fully confluent 24 to 48 hr after seeding.

For routine assay of DNA infectivity with DEAE-D, monolayers washed once with 3 ml. phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) were incubated for 15 min. at 37° with 0.1 ml. of a solution of DEAE-D (1000 µg./ml.) in PBS, then with 0.1 ml. of virus DNA diluted in PBS for 30 min. at 37° with occasional tilting. Ten ml. overlay medium (Eagle's medium (× 1.3 strength) with 0.9% Difco Bacto agar and 2.5% horse serum) were added and the cultures were incubated at 37° in a CO₂ incubator for 13 days. Then 3 ml. of overlay medium containing neutral red (0.008%) were added to reveal the plaques on the fourteenth day of incubation. The procedures used in the hypertonic method of assay were those described by Weil (1961).

DEAE-D

A preparation of average molecular weight 2×10⁶ was obtained from Pharmacia Ltd, Uppsala, Sweden. Stock solutions (20 mg./ml.) were made in PBS and sterilized by autoclaving.

RESULTS

Plaque formation by DNA extracts of polyoma virus promoted by DEAE-D

Plaques visible after about 13 days were produced after infection of monolayers with dilutions of DNA extracted from unpurified virus in the presence of DEAE-D at a final concentration of 500 µg./ml. No plaques appeared when DEAE-D was omitted, or when the extract was treated with DNase (100 µg./ml. for 15 min. at 37°) before mixing with DEAE-D (Table 1).

In all subsequent experiments DNA extracted from purified virus was used.

The effect of different adsorption conditions on the plaque-forming response

The effect of mode of treatment with DEAE-D. Pre-exposure of the monolayers to DEAE-D at 37° before addition of DNA, the method routinely used, gave the same number of plaques as addition of a mixture of DNA and DEAE-D made 20 min. previously. However, exposure of the monolayers to DNA before DEAE-D reduced the number of plaques (Table 2).

The effect of temperature and time of adsorption. The influence of the temperature of adsorption on the number of plaques produced by component I was relatively
slight. After a 30 min. adsorption period, a similar number of plaques was formed at 20° and 37°, and the number was reduced only to half at 4°. At 37° the maximum plaque titre was obtained after 60 min., although the titre reached 50% of the maximum after only 5 min. (Table 3).

The effect of DEAE-D concentration. Although plaques were produced by virus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution of extract</th>
<th>No. of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ DEAE-D</td>
<td>10⁻²</td>
<td>38, 45, 55, 47</td>
</tr>
<tr>
<td></td>
<td>10⁻³</td>
<td>13, 11, 27, 22</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>4, 3, 5, 5</td>
</tr>
<tr>
<td>No DEAE-D</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DNase treated</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+ DEAE-D</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Plaque formation by DNA extracted with phenol from unpurified polyoma virus adsorbed in the presence of DEAE-D

Table 2. The effect of mode of treatment with DEAE-D on the plating efficiency of component I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-D and DNA pre-mixed</td>
<td>111, 115, 100</td>
</tr>
<tr>
<td>DEAE-D, then DNA added*</td>
<td>104, 108, 111</td>
</tr>
<tr>
<td>DNA then DEAE-D added†</td>
<td>34, 38, 41</td>
</tr>
<tr>
<td>DNA without DEAE-D‡</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

Component I was used at a dose of 10⁻³ μg. DEAE-D final concentration was 500 μg./ml.
*DEAE-D for 15 min., DNA for 30 min.
† DNA for 15 min., DEAE-D for 30 min.
‡ 30 min.

![Graph](image-url)

Fig. 1. The effect of DEAE-D concentration on the plating efficiency of component I (DNA dose 2·5 × 10⁻⁸ μg./culture).
DNA at DEAE-D concentrations as low as 5 µg./ml. DEAE-D, higher concentrations were necessary for maximum plaque titre. The response was maximal and roughly constant from 50 to 500 µg./ml. DEAE-D, but decreased outside this range (Fig. 1). Control monolayers not exposed to DNA showed evidence of slight necrosis at a concentration of 5000 µg./ml.

Table 3. The effect of time of adsorption at 37° on plating efficiency of component I in the presence of DEAE-D

<table>
<thead>
<tr>
<th>Adsorption time (min.)</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of plaques</td>
<td>0, 0, 0</td>
<td>46, 43, 51</td>
<td>76, 62</td>
<td>67, 75, 72</td>
<td>95, 86</td>
<td>74, 81</td>
</tr>
</tbody>
</table>

Component I was used at a dose of 10⁻⁴ µg. DEAE-D final concentration was 500 µg./ml.

Table 4. Comparison of plating efficiencies for component I in the DEAE-D and hypertonic methods

<table>
<thead>
<tr>
<th>Dose of DNA (µg.)</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of plaques</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertonic method</td>
<td>115, 148</td>
<td>37, 21, 27</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DEAE-D method</td>
<td>--</td>
<td>117, 96, 105</td>
<td>36, 29, 28</td>
<td>--</td>
</tr>
</tbody>
</table>

A similarly broad optimum concentration range of DEAE-D has been found for other virus nucleic acids (Vaheri & Pagano, 1965; Koch, Quintrell & Bishop, 1966). Routinely a DEAE-D concentration of 500 µg./ml. at the upper end of the optimum range was used to allow for dilution by any PBS remaining after washing monolayers.

Comparison of the DEAE-D method with the hypertonic method

The sensitivities of the DEAE-D method and the hypertonic method of Weil (1961) were compared using component I. The DEAE-D method was about 100 times more efficient (Table 4). Addition of DEAE-D at 500 µg./ml. to the hypertonic medium increased the efficiency to that obtained with DEAE-D under isotonic conditions.

Variation in sensitivity of the assay

The plating efficiency for a single DNA preparation varied for batches of monolayers prepared at different times. The extent and cause of variation have not been studied systematically, but responses as low as 1/10th to 1/100th of the normal were occasionally observed for a single preparation of component I. The relative sensitivities of the DEAE-D and hypertonic methods, however, remained roughly constant, suggesting that uncontrolled cellular factors were responsible for the variation.

Dose–response relationships for components I and (II+III)

Component I and component (II+III) obtained from purified virus were assayed for infectivity over a range of DNA concentrations by the DEAE-D method. The results for DNA extracted with sodium dodecyl sulphate are shown in Fig. 2.
Infectivity of polyoma virus DNA

At lowest DNA concentrations, the response was proportional to dose for both components but rapidly levelled off at higher concentrations. The initial slope for the component I response curve was approximately fourfold greater than that for component (II + III). The specific infectivities calculated from these slopes were $6 \times 10^5$ and $1.5 \times 10^6$ p.f.u./μg., respectively. Similar results were obtained with DNA extracted with phenol. Specific infectivities for components I and (II + III) ranged from $5 \times 10^3$ to $6 \times 10^5$ p.f.u./μg. and $1.4 \times 10^3$ to $1.5 \times 10^5$ p.f.u./μg. respectively, in assays of several different preparations.

![Dose-response curve for plaque formation of components I and (II + III) in the DEAE-D assay.](image)

**Fig. 2.** The dose–response for plaque formation of components I and (II + III) in the DEAE-D assay. The DNA was prepared by extraction with sodium dodecyl sulphate. The mean plaque titre of 4 cultures at each dose is plotted. The error bars indicate ± standard deviation. ●●●● component I; × × × component (II + III).

<table>
<thead>
<tr>
<th>Mouse DNA Dose (μg.)</th>
<th>0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of plaques</td>
<td>27, 18, 25</td>
<td>34, 20, 33</td>
<td>8, 22, 10</td>
<td>1, 3</td>
<td>2, 0, 1</td>
</tr>
</tbody>
</table>

Component I was used at a dose of $10^{-5}$ μg.

Table 5. The effect of the presence of mouse DNA on the plating efficiency of component I in the DEAE-D assay

The effect of added mouse DNA on virus DNA infectivity

To determine whether the presence of non-viral DNA would modify the plaque response different amounts of mouse DNA were mixed with virus DNA before assay. No effect was found below a dose of 0.1 μg. mouse DNA (100-fold excess of mouse DNA over virus DNA) but at higher doses of mouse DNA the response rapidly declined (Table 5).
DISCUSSION

The use of DEAE-D in plaque assays for polyoma virus DNA has several advantages over the hypertonic method. It is more efficient. The highest specific infectivity obtained for component I in the present work, $6 \times 10^6$ p.f.u./µg, is 50 times higher than the highest value reported using the hypertonic method, $1.25 \times 10^4$ p.f.u./µg., (Winocour, 1967). This corresponds to an efficiency of about $3 \times 10^{-6}$ or about $10^{-4}$ that of intact virus (Crawford, Crawford & Watson, 1962). In all comparative experiments of the present work, the efficiency of the DEAE-D method was about 100 times greater than that of the hypertonic method.

Monolayers are not exposed to adverse physiological conditions. The plaque titre is relatively independent of the time and temperature of adsorption compared to the hypertonic method. The experimental operations are much simpler. Precise control of adsorption temperature is unnecessary and operations to assist recovery of the cells from the effects of hypertonic conditions are eliminated.

A limitation of the method in its present form is the narrow range of linearity. There are probably two main contributing factors. First, plaque numbers may be underestimated because of overcrowding. Secondly, the response to virus DNA probably depends on several factors including the nature of the interaction between DNA and DEAE-D, and the interaction of both these substances, individually and in complex, with the cell; this could render the response inherently non-linear over any extended range of DNA concentration.

The relative difference in specific infectivity between components I and (II + III) found with DEAE-D is similar to that reported for the hypertonic method (Dulbecco & Vogt, 1963; Bourgaux, Bourgaux-Ramoisy & Stoker, 1965). However, the relative infectivities of components I, II and III cannot be estimated because of the unknown proportions of II and III, and the probable presence of cellular DNA (Winocour, 1967; Michel, Hirt & Weil, 1967) which would appear in component (II + III).

The magnitude of the effect of added mouse DNA does not suggest that cellular component III could significantly inhibit virus DNA infectivity. However, the possibility that particular fractions of mouse DNA which might appear as cellular component III could interfere cannot be excluded. The mechanism of inhibition by relatively high concentrations of mouse DNA is under investigation.

The high efficiency of plaque formation suggests that the DEAE-D method may be profitably applied to other DNA viruses and may also facilitate transformation of mammalian cells by virus DNA, which has so far only been demonstrated using the hypertonic method (Crawford et al. 1964).

The work was supported by grants from the Medical Research Council and the British Empire Cancer Campaign for Research.

REFERENCES


Infectivity of polyoma virus DNA


(Received 21 March 1968)