An Infectious Centres Technique for the Assay of Infectious Polyoma Virus DNA

(Accepted 1 December 1972)

Most preparations of infectious virus nucleic acids retain only about 0.1% or less of the infectivity of the original virus material. This is not necessarily the result of destruction of much of the intact nucleic acid during chemical procedures, but may be due to the insensitivity of the assay methods employed (Holland et al. 1961; Tovell & Colter, 1967). For the titration of infectious DNA obtained from polyoma virus, one of the methods in current use requires the direct plating of DNA in 0.55 M-NaCl on the monolayers of mouse embryo cells (Weil, 1961). This procedure has the disadvantage that the cell damage caused by the hypertonic NaCl renders accurate plaque counts difficult. A more recently described method (Warren & Thorne, 1968) avoids this complication by infecting monolayers in the presence of diethylaminoethylxdatran (DEAE-dextran) in an isotonic medium. The infectious centres technique of Ellem & Colter (1960) also overcomes this difficulty but the application of this method to the assay of infective polyoma DNA was unsuccessful (Weil 1961). In this communication a method is described for the titration of polyoma DNA using an infectious centres technique, the sensitivity of which is enhanced by the addition of an appropriate dilution of dimethyl sulphoxide (DMSO).

Virus stock was prepared from the LID-I strain using the method of Winocour (1963) in which virus is collected from mouse kidney tissue cultures obtained from neonatal mice infected with polyoma virus. Apart from removal of cell debris by centrifuging, the tissue culture fluid was used without further purification. Infectious DNA was prepared from the virus stock by the method of Well (1960) which was modified in that ether was removed by evaporation in vacuo whilst the preparation was kept in ice.

Both virus and DNA were assayed on secondary tissue culture monolayers obtained from trypsinized mouse embryos. These cells were grown in Basal Eagle's Medium (BEM) containing fourfold concentrations of amino acids and vitamins (Dulbecco & Freeman, 1959) and supplemented with 10% calf serum and 1 mM-sodium pyruvate (Eagle, 1959). For the assay of virus the method of Dulbecco & Freeman (1959) was used, the agar overlay containing the reinforced BEM mentioned above and supplemented with 4% horse serum instead of calf serum.

The DNA was assayed both by direct plating and by an infectious centres technique. For direct plating, the method of Weil (1961) was used with some modifications, namely monolayers were subjected to steplike increases in NaCl molarity until 0.55 M-NaCl was reached. After infection with virus DNA at this molarity and appropriate incubation, NaCl molarity was reduced in steplike fashion and finally monolayers were covered by 'recovery medium'. Suspensions of cells for the infectious centres technique were obtained by removing secondary cell sheets from bottles with 5 × 10^{-4} M-versenate in 0.04M-phosphate buffer (pH 7.2). The cells were washed in phosphate buffer, resuspended and counted. Samples containing appropriate concentrations of cells were centrifuged in conical tubes to deposit the cells, and the supernatant fluids removed. The cell deposits were resuspended in 0.5 ml of suspending medium which consisted of various concentrations of NaCl in 0.04 M-phosphate buffer. To each cell suspension 0.5 ml of infectious virus DNA was added,
Table 1. Effect of DMSO on virus DNA infectivity

<table>
<thead>
<tr>
<th>Suspension medium</th>
<th>p.f.u./ml</th>
<th>e.o.p.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.55 M-NaCl</td>
<td>106</td>
<td>8.8 x 10^-6</td>
</tr>
<tr>
<td>10% DMSO (v/v) in PBS</td>
<td>160</td>
<td>1.3 x 10^-5</td>
</tr>
<tr>
<td>15% DMSO (v/v) in PBS</td>
<td>745</td>
<td>6.2 x 10^-4</td>
</tr>
<tr>
<td>20% DMSO (v/v) in PBS</td>
<td>333</td>
<td>2.8 x 10^-5</td>
</tr>
<tr>
<td>25% DMSO (v/v) in PBS</td>
<td>13</td>
<td>1.1 x 10^-4</td>
</tr>
</tbody>
</table>

* The efficiency of plating (e.o.p.) was based on the concentration of virus in the original preparation (1.2 x 10^7 p.f.u./ml) from which DNA was prepared.

Table 2. Effect of cell concentration on formation of infectious centres by DNA

<table>
<thead>
<tr>
<th>Cell concentration</th>
<th>DNA Assay (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^7</td>
<td>2.7 x 10^4</td>
</tr>
<tr>
<td>5 x 10^8</td>
<td>5.3 x 10^4</td>
</tr>
<tr>
<td>1 x 10^8</td>
<td>1.1 x 10^5</td>
</tr>
<tr>
<td>5 x 10^8</td>
<td>6.4 x 10^5</td>
</tr>
<tr>
<td>1 x 10^7</td>
<td>1.4 x 10^6</td>
</tr>
</tbody>
</table>

the nucleic acid being in the same suspending medium as the cells. The cell-DNA mixtures were agitated for 2 min whilst kept in ice and then for a further 5 min while held in a 37 °C water bath. After incubation the mixtures were subjected to tenfold dilution in reinforced BEM containing 4% horse serum and further appropriate dilutions were made in the same medium. These cell suspensions in 3 ml volumes were placed on to fresh secondary mouse embryo monolayers in 50 mm Petri dishes drained of growth medium. Cells were allowed to settle at 37 °C in an atmosphere of 5% CO_2 in air for 3 h. After this, fluid was removed and replaced by nutrient agar overlay similar to that used for the virus assay. A further 2 to 3 ml of agar overlay was added on day 6 and 10. To the latter Neutral Red (1/20000) was added. Plaques were recorded daily until day 21.

Initial attempts at assaying infectious polyoma virus DNA in 0.55 M-NaCl by the ‘infectious centres’ technique were unsuccessful. However, by increasing the concentration of cells in the suspension to over 6 x 10^6/ml of DNA-cell mixture, plaques could be obtained on seeding this mixture on to monolayers. This method, however, gave a plaque count one third to a half of that obtained using the conventional method of direct plating onto monolayers in 0.55 M-NaCl (Weil, 1961). Since dimethyl sulphoxide (DMSO) at a concentration of 10 to 12.5%, was shown by Tovell & Colter (1967) to enhance the infectivity of Mengo virus RNA in hypertonic solutions using the infectious centres technique, the effect of DMSO on polyoma virus DNA was tested. The infectious DNA was incorporated in a mixture of hypertonic 0.55 M-NaCl solution and DMSO so that the final concentration of DMSO varied from 10 to 25% (v/v). No enhancement of DNA infectivity was obtained. DMSO incorporated in isotonic phosphate-buffered saline (PBS - 0.14 M-NaCl in 0.02 M-phosphate buffer, pH 7.2), on the other hand, was found to enhance the infectivity of infectious DNA. The optimal concentration of DMSO necessary for this enhancement was determined by exposing similar samples of cells to DNA containing varying concentrations of DMSO. After the appropriate period of incubation these samples of cells were plated out as ‘infectious centres’ on to monolayers prepared from secondary cultures of mouse embryo cells. The results of one such experiment are shown in Table 1. Maximal enhancement was seen to occur with 15% DMSO (v/v) in isotonic PBS. These findings were confirmed at this concentration of DMSO in the e.o.p. range 3.1 x 10^-6 to 2.3 x 10^-4.
Fig. 1. Infectious centres obtained by exposing the same sample of infectious DNA to successive cell samples. Two experiments are illustrated. In one, represented by the two left-hand bars, the sample was exposed to two successive samples. In the other, the sample was exposed to five successive samples. The number of infectious centres in the samples 2 to 5 is expressed as a percentage of the number produced in sample 1.

In the infectious centres method the concentration of cells used in the DNA-cell suspension is important. The effect of cell concentration was studied by exposing similar quantities of infectious DNA to varying concentrations of cells and plating out these mixtures as infectious centres. The results of one representative experiment are shown in Table 2.

It is seen that there is sharp increase in plaque formation when the cell concentration is increased above $10^6$ cells/ml of cell-DNA mixture.

In spite of the enhancing effect of DMSO, free infectious DNA may still be demonstrated in the DNA preparation after exposure to the initial cell suspension. This was demonstrated by incubating a series of cell suspensions, each containing the same number of cells (varying from $6.5 \times 10^6$ to $9 \times 10^6$ in different experiments), with a single sample of DNA. The supernatant fluid was transferred to a second cell sample, incubated for the same time as the first and plated out as infectious centres. The results of two experiments in which 2 and 5 successive cell samples were used respectively, are shown in Fig. 1. The number of infectious centres produced in cell samples 2 to 5 is expressed as a percentage of the number produced in cell sample 1. From these figures it is obvious that a large amount of infectious DNA remains in the DNA preparation after exposure to the initial suspension of cells.

Tovell & Colter (1967) showed that the infectivity of mengovirus RNA was enhanced by DMSO in the presence of hypertonic sucrose. By contrast, maximal infectivity of polyoma virus DNA was obtained with 15% DMSO (v/v) in isotonic PBS whereas no such enhancement was encountered with DMSO in hypertonic NaCl. This illustrates that conditions for maximal efficiency of infectivity for each system must be determined individually.

The number of infectious centres formed is dependent on the concentration of cells present. When more than $10^6$/ml cells are present in the cell-DNA mixture, the plaque count increases exponentially. It is important therefore, when using this method, to ensure
that not only an adequate concentration of cells is present but also that similar concentra-
tion of cells are used when comparing different preparations.

By exposing polyoma virus DNA to successive suspensions of mouse embryo cells, it was shown that free infectious DNA was demonstrable in the supernatant fluid even when high concentrations of cells were used. Similar findings were obtained by Tovell & Colter (1967) in the case of mengovirus RNA indicating that the relatively insensitive assay methods do not show up all the infectious virus nucleic acid present in preparations. Further narrowing of the gap between the infectivity of the original virus preparation and that of the infectious nucleic acid derived from it must await upon additional improvements in the technique.

We wish to thank Professor A. Kipps for his interest and encouragement in this work.

M.R.C. and U.C.T. Virus Research Unit
Medical School, Observatory, Cape, South Africa

REFERENCES


(Received 3 August 1972)