Transformation of Hamster Cells in vitro by Adenovirus Type 12

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SUMMARY

Hamster brain and kidney cells used as secondary cultures were transformed by adenovirus type 12. Transformed brain cells established in serial culture contained adenovirus tumour antigen and produced tumours when injected into hamsters. Conditions under which transformation may be assayed were determined. In such an assay up to 1/20,000 infected cells gave rise to transformed foci. At high virus input the rate of transformation was decreased probably owing to development of cytopathic effect.

INTRODUCTION

In vitro transformation of cell cultures by adenovirus type 12 has been reported from several laboratories, but transformation seems to have been a rare occurrence, and slow to appear. McBride & Wiener (1964) achieved transformation in cultures of baby hamster kidney and established a line of transformed cells in serial culture. Pope & Rowe (1964) described transformation in cultures of hamster embryo cells, and Levinthal & Petersen (1965) produced transformed foci in cultures of rabbit and rat kidney. In the latter two studies transformation was not observed until 70 or more days after inoculation of the cultures. Freeman et al. (1966a) noted transformation in a rat embryo system, and Freeman et al. (1966b) emphasized the importance of using a medium of low calcium content in transformation studies. Recently, Yamane & Kusano (1967) described transformation occurring in cultures of hamster brain. The present report concerns attempts to quantify the transformation process in hamster brain and kidney cells and to investigate some factors influencing the observed transformation rate.

METHODS

Cultures were prepared from brains of embryo or neonatal hamsters. Meninges were stripped from the brains, which were chopped finely and dispersed using 0.25% (w/v) Difco trypsin in 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7.4) with 3% (v/v) foetal calf serum. Growth medium was Eagle's minimal essential medium with twice the normal concentration of vitamins and amino acids + 10% foetal calf serum. Primary cultures of hamster kidney were prepared in a similar way, except that no serum was added to the trypsin. Secondary cultures of brain and kidney were used for transformation experiments.

A strain of adenovirus type 12, isolated in human amnion and passed only in human

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embryo kidney cells, was used (Pereira & MacCallum, 1964). Monolayers of human embryo kidney cells showing complete cytopathic effect were harvested by scraping the cells into the medium. The suspension was frozen and thawed three times and clarified by centrifugation at 2000 rev./min. for 10 min. The supernatant fluid was used as stock virus. The stocks were titrated in human embryo kidney cells, and total particle counts were made by electron microscopy by the method of Watson, Russell & Wildy (1963). The ratio of total particles to infective particles was about $10^{2.6}$. Mouse feeder cultures were prepared by the method of Stoker & Macpherson (1961), except that the X-irradiation dose was 1700 rads.

RESULTS

Transformation in monolayer cultures

Hamster brain cultures grew very slowly, the cells doubling in number in about a week. There was a diversity of cell types, many having long filamentous processes, others being smaller and more rounded (Pl. 1, fig. 1). Monolayers of secondary brain cultures were inoculated with adenovirus 12 at an approximate input multiplicity of $10 \text{TCD}_{50}$ per cell, and were maintained either on Eagle's medium + 10% tryptose phosphate broth + 10% foetal calf serum or on the same medium without calcium chloride. Medium was changed daily for the first 4 days, when some cytopathic effect was evident, and then twice weekly. After 29 days in standard medium or 33 days in low calcium medium foci of cells showing a marked change in morphology were noticed. The number of foci appearing was approximately one per $2 \times 10^{5.5}$ cells present at the time of inoculation. The foci enlarged rapidly, producing sharply defined, dense piles of cells easily identified by low-power microscopy or by naked eye examination. These foci did not develop in uninoculated control cultures; they were easily distinguishable from localized areas of piling-up of cells which occurred in both inoculated and control monolayers.

Nature of the transformed cells

Clumps of cells were picked from an abnormal focus on the monolayer using a fine Pasteur pipette and after some difficulty were established in serial culture. The most suitable medium proved to be Eagle's medium without calcium chloride, but with four times the normal amount of vitamins and amino acids + 10% tryptose phosphate broth + 10% foetal calf serum (AT medium). The calcium content of this medium was low, being dependent upon calcium present in the serum and the tryptose phosphate broth. The precise level was not determined.

The transformed cells were small, with scanty cytoplasm and large nuclei (Pl. 1,
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They grew rapidly, reaching a density of approximately $2.5 \times 10^7$ cells in a monolayer of 25 cm.$^2$. These cells have been maintained through approximately 50 subcultures over a period of 5 months with no diminution of their growth rate. The growth rate of untransformed brain cells declined steadily; they could not be maintained beyond 9 or 10 subcultures. The transformed cells produced tumours when injected subcutaneously into newborn or weanling hamsters (Table 1).

Transformed cells were stained by the indirect fluorescent antibody method for the presence of adenovirus tumour antigens (Coons, 1958). Sera from hamsters bearing tumours induced by inoculation of adenovirus 12 were used, followed by rabbit anti-hamster-globulin serum conjugated with fluorescein. The stained cells showed one to four or more rather large, elongated flecks of specific fluorescence in the cytoplasm. Nuclei showed much fainter staining in the form of fine threads and flecks. Controls included untransformed brain cells similarly stained, and transformed cells treated with normal hamster serum followed by fluorescein-conjugated anti-hamster-globulin serum; neither showed specific staining.

Transformed cells were suspended in phosphate buffered saline at $10^7$ cells per ml., frozen and thawed twice and centrifuged at 10,000 rev./min. for 30 min. The supernatant fluid, when diluted 1/4, fixed complement with a 1/40 dilution of pooled sera from hamsters bearing tumours induced by injection of adenovirus 12. There was no fixation with 1/10 or 1/40 dilutions of pooled normal hamster sera. A control antigen similarly prepared from the hamster kidney line BHK 21/Cl 13 (Macpherson & Stoker, 1962) did not fix complement with either of the hamster serum pools.

In an attempt to reveal release of infectious virus from transformed cells, these cells were inoculated on to semi-confluent secondary cultures of human embryo kidney. The mixed cultures were observed for 2 weeks, during which time no cytopathic effect developed in the kidney cells. The transformed cells grew well under these conditions, eventually forming a monolayer superimposed on the human cells.

Quantitative assay of transformation

In an attempt to quantify the transformation process experiments were made by a method based on a polyoma virus transformation assay using feeder layers of X-irradiated mouse embryo cells (Stoker & Abel, 1962). Preliminary observations on the growth of transformed cells at low densities indicated that their growth was enhanced by high densities of X-irradiated mouse feeder cells. Feeder layers of mouse cells were therefore prepared by inoculating 60 mm. Petri dishes each with $10^6$ X-irradiated mouse embryo cells. The semi-confluent cultures which resulted were used as feeder layers.

Hamster brain or kidney cells were infected by mixing them for 2 hr at 37$^\circ$ with crude adenovirus 12 suspension, then resuspending them in AT medium and seeding them on the feeder layers. Cultures were fed three times weekly and were fixed and stained after about 2 weeks. Foci of transformed cells were counted at $\times 40$ magnification. The transformation rate was expressed as the logarithm of the ratio of the number of transformed foci counted to the total number of infected cells seeded (t/cell). The effect of varying the number of infected cells seeded was studied at three different incubation temperatures. Brain cells were infected in suspension at a virus-to-cell ratio of approximately 1 TCD 50 per cell. Cells were then centrifuged, counted, suspended at three different concentrations in AT medium and seeded on feeder layers.
layers, using twelve replicate Petri dishes for each cell density. Groups of four Petri dishes at each density were incubated at 37°, 34° or 31° for 1 week, then at 37° for a further 11 days to allow any cells transformed at the lower temperatures to form detectable foci. In the presence of the thick feeder layers used, the highest transformation rates were obtained when the number of adenovirus-infected hamster cells added to each Petri dish was relatively small (Table 2). If larger numbers of hamster cells were seeded they formed a thick, confluent layer superimposed on the feeder cells; the observed rate of transformation was then somewhat lower. Of the tempera-

Table 2. Effect of variation of number of infected cells plated and of incubation temperature on rate of transformation of hamster brain cells

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Brain cells per Petri dish</th>
<th>Transformed foci per Petri dish</th>
<th>t/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1 × 10⁵</td>
<td>17, 17, 12, 17</td>
<td>10⁻⁴.⁸</td>
</tr>
<tr>
<td>37</td>
<td>2 × 10⁵</td>
<td>13, 6, 5, 8</td>
<td>10⁻⁴.¹</td>
</tr>
<tr>
<td>37</td>
<td>4 × 10⁴</td>
<td>1, 3, 3, 1</td>
<td>10⁻⁴.⁸</td>
</tr>
<tr>
<td>34 → 37</td>
<td>1 × 10⁶</td>
<td>5, 1, 1</td>
<td>10⁻⁵.⁸</td>
</tr>
<tr>
<td>34 → 37</td>
<td>2 × 10⁵</td>
<td>3, 2, 1, 1</td>
<td>10⁻³.³</td>
</tr>
<tr>
<td>34 → 37</td>
<td>4 × 10⁴</td>
<td>0, 0, 0</td>
<td>—</td>
</tr>
<tr>
<td>31 → 37</td>
<td>1 × 10⁶</td>
<td>0, 1, 1, 0</td>
<td>10⁻⁵.³</td>
</tr>
<tr>
<td>31 → 37</td>
<td>2 × 10⁵</td>
<td>0, 0, 0, 0</td>
<td>—</td>
</tr>
<tr>
<td>31 → 37</td>
<td>4 × 10⁴</td>
<td>0, 0, 0, 0</td>
<td>—</td>
</tr>
<tr>
<td>(Uninoculated)</td>
<td>1 × 10⁴</td>
<td>0, 0, 0, 0</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of variation of virus input on rate of transformation (t/cell) of hamster brain cells.
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tures investigated, continuous incubation at 37° gave the best rates of transformation; this temperature was used for subsequent experiments.

The effect on the transformation rate of variation of virus dose was investigated. Samples of brain cells were infected in suspension using stock adenovirus 12 diluted in tris buffer to give approximate input multiplicities of 4, 2, 1 and 0.5 TCD50 per cell. The experiment was repeated using slightly lower virus doses and brain cells from another litter of hamsters. Similar results were obtained (Fig. 1). Clearly, the maximum transformation rate was not obtained with the highest dose of virus. This observation may perhaps be explained by the fact that at the higher input multiplicities some cytopathic effect was evident, maximal on the third or four day after inoculation. The degree of cell destruction was related to virus dose, being maximal at the highest input and negligible at the lowest.

Table 3. Transformation of secondary cultures of baby hamster kidney by adenovirus type 12

<table>
<thead>
<tr>
<th>Approx. multiplicity (TCD 50/ cell)</th>
<th>Target cells per Petri dish</th>
<th>Transformed foci per Petri dish</th>
<th>t/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brain 1×10^4**</td>
<td>13, 16, 5, 8</td>
<td>10^-1</td>
</tr>
<tr>
<td>0</td>
<td>Kidney 1×10^5**</td>
<td>0, 0, 0</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>Brain 1.7×10^5**</td>
<td>3, 1, 1, 0</td>
<td>10^-1</td>
</tr>
<tr>
<td>0</td>
<td>Kidney 5×10^4**</td>
<td>0, 0, 0</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Brain 4×10^6**</td>
<td>18, 11, 11, 10</td>
<td>10^-5</td>
</tr>
<tr>
<td>0</td>
<td>Kidney 2×10^6</td>
<td>0, 0, 0</td>
<td>---</td>
</tr>
</tbody>
</table>

* Brain and kidney cells prepared from the same embryos.

Several experiments were made using secondary cultures of hamster kidney as the target cell. In one of these experiments t/cell values obtained at a virus-to-cell ratio of 1 were compared using brain and kidney cells prepared from the same batch of embryos (Table 3). Foci of transformed cells in kidney cultures were morphologically indistinguishable from foci of transformed brain cells (Pl. 1, fig. 3).

DISCUSSION

A minimum of 10^4-5 infective particles, or, with the stock virus used, 10^7-1 physical particles was necessary to produce one observable transformed focus. The transformation process was therefore inefficient in comparison with the polyoma virus + hamster cell system in which 1 in 10^3 infective particles or 1 in 10^5 physical particles may cause transformation (Stoker & Abel, 1962; Macpherson & Montagnier, 1964). Further modifications of the conditions of the test might improve the observed rate of transformation. Despite use of an enriched medium, transformed cells grew poorly at low densities, though this situation could be improved by the use of dense layers of feeder cells. However, the presence of too many untransformed cells lowered the rate of detection of transformed foci. This effect may be partly due to difficulty in identification of transformed foci in a dense monolayer, and perhaps partly to contact inhibition of the transformed cells (Stoker, 1964). Attempts to increase the transformation rate by use of a high virus input were complicated by development of cytopathic effect. The observed reduction of the transformation rate at high virus input may have
indicated that target cells capable of being transformed are also susceptible to destruction by adenovirus 12. Cytopathic effect occurring early in the course of adenovirus infection may be due to the penton antigen (B antigen, early cytopathic factor) (Pereira, 1958). In the present instance, however, cell destruction was delayed, being maximal on the third and fourth days. Moreover, Huebner et al. (1964) noted that prototype adenovirus 12 when fractioned on DEAE-cellulose did not produce B antigen. Use of purified virus in transformation studies might clarify the role of the penton antigen in this context.

Brain and kidney cells from different batches of hamsters showed considerable variation in response to adenovirus 12 under apparently identical conditions, making impossible any direct comparison of the one experiment with another. However, in one experiment brain cells and kidney cells prepared from the same embryos, infected at a virus-to-cell ratio of 1 and plated at similar densities, gave t/cell values of $10^{-4.4}$ and $10^{-5.1}$ respectively at the single input multiplicity tested. Yamane & Kusano (1967) considered that the target cell for adenovirus transformation was of nervous origin. The present data are insufficient to suggest the precise origin of the target cell, but indicate that such cells occur not only in brain cultures, but also in kidney cultures at a rate of not less than $10^{-4.5}$. Use of a target cell which grows slowly is one of the conditions of the present test which selectively favours identification of transformed foci. A rapidly growing cell such as BHK21/C13 produces a thickly overgrown monolayer in the 2-week period required for development of transformed foci. Transformation of this cell line was not, in fact, detected by the present technique.

I should like to thank my colleagues at the Institute of Virology, and in particular Professor Michael Stoker, for much helpful advice and discussion. I am also indebted to Mr Augustin Okeke and Mrs Martha Macnamara for able technical assistance, to Mr M. R. Young of the National Institute for Medical Research for the photographs, and to Mrs Marguerite Pereira for the strain of adenovirus type 12 used. The work was supported by the Medical Research Council.

REFERENCES

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EXPLANATION OF PLATE

Fig. 1. Secondary culture of hamster brain cells. Giemsa stain. × 260.
Fig. 2. Adenovirus-transformed hamster brain cells. Giemsa stain. × 260.
Fig. 3. Focus of transformed cells developing in adenovirus-inoculated secondary hamster kidney culture, seeded on feeder layer of X-irradiated mouse cells. Foci of transformation in brain cultures were virtually indistinguishable from foci in kidney cultures. Giemsa stain. × 50.