Oncogenic Transformation of Rat Embryo Fibroblasts with Photoinactivated Herpes Simplex Virus: Rapid In Vitro Cloning of Transformed Cells

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SUMMARY

Rat embryo fibroblasts (REF) were transformed in vitro with photoinactivated herpes simplex virus. Low passage (7 to 10) HSV-transformed rat cells (t-REF-line G) produced multiple tumours in 49% of newborn rats with a latent period of 20 to 24 weeks. An in vitro cloning procedure for transformants in the uncloned t-REF-line G cells produced clonal lines which varied from non-oncogenic to clonal lines producing tumours with shorter latent periods (10 to 14 weeks) compared to uncloned cells. At passage 30, t-REF-line G-clone I cells produced rapidly growing tumours in 100% of the newborn rats with a latent period of only 2 to 3 weeks. Tumour cells (RSF 12-22-75) established in culture produced tumours within 2 weeks after subcutaneous (s.c.) inoculation of weanling rats (100% with tumours) and they were transplantable to 100% of inoculated adult rats. Histopathological examination of all tumours produced in newborn, weanling or adult rats revealed large, poorly differentiated malignant fibrosarcomas; metastatic tumours were observed in the lungs of 10 to 20% of newborn rats inoculated s.c. with RSF cells. Approx. 25 to 50% of the clonal transformed or tumour cells synthesized HSV-specific-antigens detected by immunofluorescence. HSV-transformed and tumour cells are resistant to superinfection by the homologous transforming virus. Since the in vitro cloning procedure for transformant cells can readily segregate cells producing clonal lines varying in oncogenic potential, the procedure might have useful application in elucidating HSV oncogenesis.

INTRODUCTION

The cell transformation potential of herpes simplex virus, type 2 (HSV-2) was first reported by Duff & Rapp (1971) in rodent cells after infection with ultraviolet light-inactivated virus. Subsequently, transformation of mammalian cells was demonstrated with photoinactivated-HSV-2 (Rapp, Li & Jerkofsky, 1973), or temperature sensitive virus mutants (Macnab, 1974; Kimura et al. 1975). In addition, Darai & Munk (1973) and Munk & Darai (1975) reported transformation of human cells and rat cells with active HSV at 42°C. We used photoinactivated virus to transform human cells at a temperature (42°C) non-permissive for virus replication (Kucera & Gusdon, 1976). Of possible significance
are data showing that HSV-2 stimulated human cell DNA synthesis during incubation at 42 °C (Melvin & Kucera, 1975; Marcon & Kucera, 1976).

Because of the oncogenic potential of photoinactivated HSV, an investigation was conducted to delineate some of the virological, immunological and oncogenic properties of cells transformed by photoinactivated virus. Results from this study provide the first evidence for malignant transformation of rat embryo fibroblasts by photoinactivated HSV. Using an in vitro procedure for cloning transformants, it was possible to clone HSV-transformed cells at low passage levels and segregate the clones for oncogenicity, producing clones which are non-oncogenic or more oncogenic than the parental cell line.

METHODS

Source of tissue culture cells and medium. Rat embryo fibroblasts (REF) were obtained from 18-day-old rat embryos (syngeneic White Buffalo strain). The rat colony was maintained as an inbred colony by sister-brother matings at the Bowman Gray School of Medicine, Winston-Salem, N.C. The REF, HSV-transformed REF, tumour cell lines and human embryonic fibroblasts (HEF, mycoplasma free; Flow Labs, Rockville, Md.) were grown in Eagle's minimal essential medium (E-MEM; Flow Labs, Rockville, Md.) or M-199 (Grand Island Biological Co., Inc., Grand Island, N.Y.).

Virus and virus assay. Strain ANG of HSV was obtained from L. Falk, Rush-Presbyterian-St. Luke's Hospital, Chicago, Ill. This strain has been used to transform human cells (Darai & Munk, 1973; Kucera & Gusdon, 1976). Recent evidence suggests that strain ANG is closely related to type 1 of HSV (Darai & Munk, 1976). The virus was grown and assayed in HEF monolayers as previously described (Melvin & Kucera, 1975). The HSV-1 (Hill strain) and rabbitpox virus were grown and assayed in HEF cells as described above for HSV (ANG strain).

Neutral red dye (NR)-light inactivation (photoinactivation) of virus. The method for photoinactivation of HSV was published by Rapp et al. (1973). Briefly, confluent HEF culture monolayers in 75 cm² flasks (Falcon Plastics) were treated with 25 μg neutral red/ml of E-MEM growth medium for 2 h at 35 °C. The medium was decanted, the cells were washed four times with 0-025 M-tris (hydroxymethyl) aminomethane (tris)-HCl-buffered saline, pH 7·0 (TB), and infected with HSV (m.o.i. = 0·5). After a 1·5 h virus adsorption period at 28 °C, the unadsorbed virus was removed by washing the cells twice with TB. The washed cultures were overlaid with E-MEM growth medium, covered with aluminium foil to exclude light, and incubated at 35 °C. Mock-infected cultures were treated in the same manner as HSV infected cultures. After 3 days' incubation, when more than 75% of the HSV infected cells showed virus cytopathology, all cultures were frozen and thawed three times. The cell suspensions were sonicated at full power (Branson sonifier) and the clarified supernatants (1000 g, for 10 min) containing virus stocks were stored at −85 °C in 2 ml samples. The titre of virus stocks measured by plaque assay in HEF cell monolayers ranged from 10⁷ to 10⁸ p.f.u./ml before photoinactivation.

Virus stocks were photoinactivated immediately before the start of a transformation experiment. To photoinactivate virus, a sample of frozen virus was rapidly thawed, sonicated, diluted tenfold in TB and exposed to 2 fluorescent bulbs (General Electric F15T8B, 15 watt, blue fluorescent) at a distance of 5 inches for 18 min with constant shaking. All virus suspensions were protected from extraneous light before or after photoinactivation. Supernatants from mock-infected cells were treated in identical manner to clarified supernatants from virus infected cells.
In vitro cloning of HSV transformed cells. A modification (Kucera & Simonson, 1974) of MacPherson's agar suspension technique (MacPherson, 1969) was used for cloning HSV-transformed and control cells. Briefly, 2 ml of basal medium, consisting of E-MEM growth medium supplemented with 2 × concentrations of Eagle's vitamins and amino acids (North American Biologicals, Inc.) and 0·5% Noble agar was overlaid with 1 × 10^4 to 1 × 10^5 mock-infected REF or HSV-transformed cells (passage 4 to 7) suspended in 4 ml of the same basal medium but with 0·3% Noble agar. The cultures were incubated at 35 °C for 2 to 3 weeks and observed for colony formation by light microscopy. Individual cell colonies were picked using a capillary pipette, suspended in 2 ml of E-MEM growth medium and incubated at 35 °C. Cell colonies which grew to confluence were serially passaged in tissue culture to establish clonal cell lines.

Indirect immunofluorescence test. Hyperimmune rabbit antiserum raised against HSV-infected rabbit kidney cell antigens was prepared as previously described (Kucera & Gusdon, 1976). Each 1 ml of serum was absorbed with 1 × 10^8 sheep red blood cells and then with 1 ml of foetal calf serum at 37 °C for 1 h to remove non-specific antibodies to heterophile antigens and serum respectively. HSV-specific antigens were detected with fluorescein-conjugated goat anti-rabbit IgG serum (Duff & Rapp, 1971). Briefly, coverslip cultures were washed three times with ice-cold 0·01 M-phosphate buffered saline, pH 7·0 (PBS), air-dried, fixed for 10 min in acetone at −20 °C and treated with a 1:2 dilution of heated (56 °C, 30 min) antiserum at 37 °C for 30 min in a humidified atmosphere. The treated coverslip cultures were washed three times with PBS and overlaid with anti-rabbit conjugate for 30 min at 37 °C in a humidified atmosphere. The washing process was repeated. The coverslip cultures were mounted in glycerol and the cells were examined without prior knowledge of specimen identity using a Zeiss microscope with an u.v. light source.

Tests for oncogenicity. Cultures to be tested for oncogenicity were trypsinized with 0·25 % trypsin-0·05 % EDTA. The detached cells were washed twice with E-MEM growth medium and twice with PBS. One-tenth ml of PBS containing 1 × 10^6 cells was inoculated subcutaneously (s.c.) and intraperitoneally (i.p.) or only s.c. into syngeneic rats. Production of palpable tumours was determined daily. A necropsy was performed on all moribund animals.

In vitro preparation of tumour cell lines. Tissue was excised from primary s.c. tumours using aseptic technique. The tumours were trimmed of normal and necrotic tissue, further subdivided into small fragments and treated with 0·25 % trypsin and 0·05 % EDTA in PBS at 37 °C with constant stirring. At 30 min intervals, the cell suspension was harvested, and the cells were washed twice by centrifuging (350 g for 5 min) with E-MEM growth medium. The washed cells were pooled, counted and 1 × 10^6 cells were suspended in 4 ml E-MEM growth medium and plated in 60 mm Petri dishes. The culture dishes were incubated at 35 °C in a humidified atmosphere containing 5% CO₂. The cultures were serially passaged when the cells reached confluence.

Histopathology of tumour tissue. Tumour tissue was fixed with 10 % formalin in PBS, sectioned and stained with haematoxylin and eosin (Kucera & Simonson, 1974).

Electron microscopy. Cells were harvested, stained and examined in a Zeiss EM 9S-2 electron microscope as previously described (Kucera & Simonson, 1974).
RESULTS

Photoinactivation of HSV

Rapp et al. (1973) reported a 5 to 6 log decrease in HSV infectivity following 12 min of light treatment of virus grown in the presence of neutral red. Similar experiments were designed to measure photoinactivation of HSV grown in human embryonic fibroblasts (HEF) pre-treated with neutral red. The virus suspension was exposed to light for varying times and residual virus infectivity was assayed in HEF cells. Results showed linear inactivation of the virus with time (Fig. 1); a 5 to 6 log decrease in virus infectivity was measured with 18 min of light exposure. Viruses which replicated in HEF cells without neutral red were not inactivated after 30 min exposure to the light (Fig. 1).

Morphological transformation of rat embryo fibroblasts (REF) with photoinactivated HSV

To transform REF cells with photoinactivated HSV approx. $5 \times 10^6$ REF cells were suspended in 20 ml of TB containing photoinactivated virus at an equivalent m.o.i. of 0.5.
Fig. 2. Focus of morphologically altered cells in a rat embryo fibroblast cell monolayer after infection with photoinactivated HSV.

(calculated before photoinactivation). The controls consisted of REF cells mock-infected with virus diluent or with clarified supernatants from uninfected HEF cells treated with the neutral red and light regimen used for preparation and photoinactivation of HSV (see Methods). The cells in suspension were sealed in aluminium foil, gently shaken for 2.5 h at 28 °C, pelleted (460 g, 4 min), washed once with M-199 growth medium and seeded in 60 mm Petri dishes (2 x 10^6 cells/5 ml M-199 growth medium/dish). All cultures were covered with aluminium foil to prevent entry of extraneous light prior to virus penetration into the cells and incubated at 35 °C in a humidified atmosphere containing 5 % CO₂. The medium was replaced at 3-day intervals with E-MEM growth medium during the 30 days post-infection. Upon reaching confluency, the cells were detached from the growing surface with 0.25 % trypsin-0.05 % EDTA in 0.01 M-PBS, pH 7.0, and passaged in 60 mm Petri dishes and successively into 25 cm² and 75 cm² plastic tissue culture flasks.

Foci of morphologically transformed rat embryo fibroblasts (abbreviated t-REF) were observed in about 10 out of 25 Petri dishes approx. 4 weeks after infection of the cells with photoinactivated HSV (Fig. 2). The foci were clearly distinguished from the cell monolayer by their loss of contact inhibition and transformed morphology. The transformed cells were smaller in size and appeared to be fibroblastoid; a few cells were epithelioid. Serially passaged transformed cells grew to a much higher saturation density (average density = 4.0 x 10^5 cells/cm²) in tissue culture compared to mock-infected non-transformed cells (average density = 6.0 x 10^4 cells/cm²). Of the ten cultures showing transformed foci, only
Table 1. Incidence and latent period for tumour formation in syngeneic rats inoculated with $1 \times 10^6$ non-transformed rat embryo fibroblasts (REF), uncloned or cloned HSV-transformed (t-REF), and tumour (RFS) cell lines*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Passage level</th>
<th>Age of host</th>
<th>Route of inoculation</th>
<th>No. of rats with tumours</th>
<th>Latent period (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-transformed REF</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>0/25 (0)</td>
<td>20–24</td>
<td></td>
</tr>
<tr>
<td>Uncloned t-REF Line G</td>
<td>7</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>24/49 (49)</td>
<td>10–16</td>
</tr>
<tr>
<td>Cloned t-REF Line G, Clone 1</td>
<td>9</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>4/8 (50)</td>
<td>1–2</td>
</tr>
<tr>
<td>Cloned t-REF Line G, Clone 2</td>
<td>7</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>0/9 (0)</td>
<td>1–2</td>
</tr>
<tr>
<td>Cloned t-REF Line G, Clone 3</td>
<td>7</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>12/13 (92)</td>
<td>10–14</td>
</tr>
<tr>
<td>Cloned t-REF Line G, Clone 1</td>
<td>30</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>28/28 (100)</td>
<td>2–3</td>
</tr>
<tr>
<td>RFS (12-22-75)</td>
<td>7–9</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>7/7 (100)</td>
<td>1–2</td>
</tr>
<tr>
<td>RFS (01-08-76)</td>
<td>2</td>
<td>Newborn</td>
<td>s.c. &amp; i.p.</td>
<td>13/13 (100)</td>
<td>3–4</td>
</tr>
<tr>
<td>RFS (12-22-75)</td>
<td>11</td>
<td>Newborn</td>
<td>s.c. only</td>
<td>7/7 (100)</td>
<td>1–2</td>
</tr>
<tr>
<td>RFS (12-22-75)</td>
<td>11</td>
<td>Newborn</td>
<td>i.p. only</td>
<td>7/7 (100)</td>
<td>1–2</td>
</tr>
<tr>
<td>RFS (12-22-75)</td>
<td>7</td>
<td>Weanling</td>
<td>s.c. &amp; i.p.</td>
<td>12/13 (92)</td>
<td>2–3</td>
</tr>
<tr>
<td>RFS (12-22-75)</td>
<td>11</td>
<td>Adult</td>
<td>s.c. only</td>
<td>13/13 (100)</td>
<td>1–2</td>
</tr>
</tbody>
</table>

* See Methods.
† Numbers in parenthesis indicate the percent of inoculated rats which developed tumours.

three (designated E, F, and G) could be serially passaged more than 10 times in tissue culture while the remaining cultures with foci entered into a crisis from which they did not recover. These results were reproducible in three independent experiments.

In vitro procedure for cloning HSV-transformed REF cells

Other investigators used continued in vivo (Duff et al. 1974) or in vitro (Kimura et al. 1975) passage of HSV transformed cells to select more oncogenic cells so that fewer cells were subsequently needed to produce tumors in inbred animals. To clone transformants, each transformed cell line (designated t-REF, line E; line F; line G) or mock-infected REF cells (passage 4 to 6) were suspended in soft-agar medium in Petri dishes and incubated at 35 °C (see Methods). Results showed that only the t-REF, line G cells grew into cell colonies which could become established clonal cell lines. The efficiency of cell colony formation for t-REF, line G transformed cells averaged 63 colonies per $1 \times 10^4$ cells.

Oncogenicity of uncloned and cloned HSV-transformed REF cells

The oncogenic potential of uncloned HSV-transformed rat embryo fibroblasts (designated t-REF, line G) and three clonal lines (designated t-REF, line G, clone 1; clone 2; clone 3) at low (7 to 9) or high (30) passage level were tested by s.c. and i.p. inoculation of syngeneic rats. Results showed that non-transformed REF cells do not produce tumors in newborn rats after an observation period of more than 12 months (Table 1). However, the uncloned parental line of transformed cells (t-REF, line G) were tumorigenic in newborn rats (49% with tumours, latent period of 20 to 24 weeks). Of the three clonal transformed cell lines
tested at low passage (7 to 9) one (t-REF-G-2) was non-oncogenic. In contrast, t-REF-G-1 and t-REF-G-3 cells were oncogenic (Table 1); the tumour incidence was 50 and 92%, respectively, with latent periods of 10 to 16 weeks. When t-REF-line G-clone-1 cells were passaged 30 times in vitro, the cells were extremely oncogenic in newborn rats; 100% with tumours and a latent period of only 2 to 3 weeks (Table 1). This is considerably faster than with passage 9 cells. In conclusion, cloning of the original parent cell line of HSV transformed rat cells in soft agar medium yielded both non-oncogenic clones and oncogenic clones with varying potentials to produce tumours with a shorter latent period than the parent line.

All s.c. and i.p. inoculated rats which developed palpable tumours also presented pearly white lesions in the peritoneal cavity involving the liver, spleen, kidneys, bladder, lymph nodes, intestines, and diaphragm. Histological examination of tumour tissue revealed extensive infiltration of tumour cells into the surrounding muscle tissue and around the spinal cord. Some primary s.c. tumours produced after inoculation of t-REF-line G-clone 1 (passage 30) cells were excised, trypsinized and propagated into tumour cell lines (designated rat fibrosarcoma, RFS 12-22-75 and RFS 01-08-76). Morphologically, the tumour cell lines cannot be distinguished from HSV clonal transformed cell lines (Fig. 3). Both RFS 12-22-75 and RFS 01-08-76 cells were highly oncogenic when transplanted into newborn syngeneic rats (Table 1). The marked oncogenic potential of RFS 12-22-75 cells was demonstrable when newborn rats were inoculated either s.c. or i.p., and when weanling rats were inoculated both s.c. and i.p. (Table 1). Twenty percent of the newborn rats inoculated s.c. with RFS 12-22-75 cells developed lung metastasis determined by histological examination of the tissues. Inoculation of RFS 12-22-75 cells into adult rats produced tumours in 13 out of 13 animals with a latent period of only 1 to 2 weeks (Table 1).
Histologically, tumours produced in newborn, weanling or adult rats inoculated with uncloned t-REF-line G, t-REF-line G-clone 1 or RFS 12-22-75 cells are composed of large, poorly differentiated, neoplastic cells (Fig. 4). Metastatic lesions in the lungs and pericardium showed the same large neoplastic cells as primary tumours. The tumour sections revealed cells in mitosis (Fig. 4), a property associated with rapidly growing tumour cells. In conclusion, the tumours were classified as malignant fibrosarcomas.

**Presence of HSV-specific antigens in transformed and tumour cells**

Persistence of HSV genomes in transformed cells is supported by antigen expression in the cytoplasm and on the membrane of these cells (Duff & Rapp, 1973; Reed, Cohen & Rapp, 1975). The reactivity of rabbit anti-HSV serum against non-transformed REF, t-REF-line G-clone 1, and RFS 12-22-75 cells was determined by indirect immunofluorescence tests. The HSV antiserum was reactive against REF cells productively infected with HSV (data not shown). When HSV antiserum was reacted with t-REF-line G-clone 1 (Fig. 5a) and RFS 12-22-75 (Fig. 5b) the typical fluorescent pattern was cytoplasmic, predominantly perinuclear fluorescence. The serum reactivity varied among different experiments and different fields; the mean positive reactivity was 25 to 50 % of the cells revealing fluorescence. The HSV antiserum produced a dull fluorescence with non-transformed REF cells (Fig. 5c).
Fig. 5. Immunofluorescence photomicrograph of HSV-transformed, tumour and non-transformed rat cells reacted with hyperimmune rabbit anti-HSV serum. (a) HSV-transformed (t-REF-line G-clone 1, passage 37) cells showing positive cytoplasmic and perinuclear fluorescence. (b) Tumour (RFS 12-22-75, passage 19) cells showing positive cytoplasmic and perinuclear fluorescence. (c) Non-transformed (REF, passage 8) rat cells showing dull fluorescence.
Table 2. Efficiency of plating (e.o.p.) of HSV-1, HSV(ANG) and rabbitpox viruses in non-transformed REF, HSV-transformed t-REF and tumour cells*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Efficiency of plating*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-transformed REF (passage 22)</td>
<td>100.0</td>
</tr>
<tr>
<td>Transformed t-REF-G-I (passage 37)</td>
<td>83.9</td>
</tr>
<tr>
<td>Tumour RFS 12-22-75 (passage 19)</td>
<td>24.2</td>
</tr>
<tr>
<td>HSV-1</td>
<td>100.0</td>
</tr>
<tr>
<td>HSV(ANG)</td>
<td>100.0</td>
</tr>
<tr>
<td>Rabbitpox</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* The e.o.p. of HSV-1, HSV(ANG) and rabbitpox viruses was determined by infecting four separate cultures of each cell type with approx. 200 p.f.u. of virus. Following 2 h virus attachment at 28 °C, all cultures were overlaid with 4 ml of E-MEM supplemented with 0.5 % methylcellulose and incubated at 35 °C for 2 days. The cultures were stained with neutral red to reveal the virus plaques. The e.o.p. is defined as the average number of plaques on HSV transformed or tumour cells divided by the average number of plaques on non-transformed cells x 100.

These data suggest persistence of HSV gene expression in the transformed and tumour cells.

Efficiency of plating (e.o.p.) of viruses in transformed and tumour cells

Dollar, Duff & Rapp (1973) reported reduced plating efficiency of HSV-1 and HSV-2 in herpesvirus-transformed hamster embryo fibroblasts, indicating resistance of the transformed cells to superinfection by the homologous transforming virus. To determine whether HSV-transformed rat cells are sensitive to superinfection, comparisons of e.o.p. of HSV(ANG) HSV-1 and a control DNA virus (rabbitpox) were carried out simultaneously at 35 °C in monolayers of non-transformed REF, HSV(ANG) transformed and tumour cells. The e.o.p. for HSV(ANG) in transformed and tumour cells was 29 and 9 %, respectively, of the e.o.p. in non-transformed cells (Table 2). Likewise, the e.o.p. for HSV-1 in transformed and tumour cells was reduced to 84 and 24 %, of the e.o.p. in non-transformed cells. In contrast, the e.o.p. for rabbitpox virus was about the same in non-transformed, HSV(ANG) transformed and tumour cells (Table 2).

Although each cell type supports virus plaque formation, the transformed and tumour cells are much less efficient in supporting HSV-1 or HSV(ANG) plaque formation.

Lack of evidence for C-type RNA viruses in transformed and tumour cells

C-type retroviruses have been detected in some HSV-2-transformed cells (Duff & Rapp, 1975; Munk & Darai, 1975). To determine the possible presence of C-type viruses in HSV-transformed (t-REF-line G-clone 1, passage 30) or tumour (RFS 12-22-75, passage 7) cells, cultures were incubated with E-MEM growth medium supplemented with 3H-uridine for 18 h at 35 °C. C-type RNA particles were not detected by measuring uptake of radioactive uridine into particles with a density of 1.15 to 1.18 g/ml after density centrifugation (Duff & Rapp, 1975) in sucrose gradients.

The two cell lines (t-REF-line G-clone 1, passage 30 and RFS 12-22-75, passage 7) were also examined by electron microscopy for budding C-type virus particles. There were no detectable C-type particles.
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DISCUSSION

The results of this investigation indicated that photoinactivated HSV can induce malignant potential in rat embryo fibroblasts (Table 1). The retention of HSV gene sequences was demonstrated by immunofluorescence of HSV-specific antigens in tumour cells (Fig. 5). Also, HSV efficiency of plating in transformed and tumour cells was significantly reduced (Table 2). These latter data corroborate published results indicating resistance of HSV-transformed hamster cells to superinfection by the homologous transforming virus; the mechanism of HSV inhibition in transformed hamster cells seems to involve some late event in virus replication (Dollar et al. 1973).

The *in vitro* procedure, employed for cloning transformants, segregated clonal cell lines (Table 1) ranging from non-oncogenic to more oncogenic than the original uncloned parent cell line. So far, one clonal line (t-REF-line G-clone 1) carried to high passage level (30) produced marked oncogenicity (100 % tumour incidence) with a significantly shorter latent period for tumour production (2 to 3 weeks) compared to low passage cells (10 to 12 weeks; Table 1). Although oncogenicity of the transformed cells increased as a function of passage level the cells were tumorigenic even at low passage (passage 7 to 9; Table 1). This observation is in contrast to two published reports (Macnab, 1974; Kimura et al. 1975) indicating that HSV-2 transformed hamster cells required extensive cultivation *in vitro* before becoming oncogenic *in vivo*. The differences between oncogenic potential of HSV transformants in our investigation and other published work are possibly due to the rapid *in vitro* cloning procedure we employed for transformant cells.

Although HSV-transformed or tumour cells synthesized virus specific antigen(s) which reacted by immunofluorescence with rabbit antiserum to HSV (Fig. 5), virus neutralizing antibodies were not detected in the sera from tumour bearing rats (L. S. Kucera, & I. Edwards, unpublished data). These two properties were present in hamster cells transformed by u.v.-inactivated HSV-1 (Duff & Rapp, 1971, 1973). In contrast, tumours produced in hamsters after inoculation of hamster cells transformed by photoinactivated HSV-2 synthesized virus-specific antigen(s) reactive with sera from tumour-bearing hamsters but not reactive with rabbit anti-HSV sera (Li, Jerkofsky & Rapp, 1975). These apparent discrepancies between our data and published data suggest (1) that different gene products are expressed after transformation by virus inactivated by u.v. light and neutral red dye-light, (2) each transformed cell line differs in the amount of virus genetic information present or expressed, (3) tumour bearing rats do not produce antibodies against HSV antigens which are present in HSV-transformed cells, or (4) the quantity of virus antigen(s) may be too small for detection or may be repressed or lost in some transformed cell lines. There are recent published reports that HSV antigen synthesis (Li et al. 1975) and detectable virus DNA sequences (Minson et al. 1976) can be lost after high passage of HSV-transformed hamster cells *in vitro*.

In our experiments, infectious HSV was not detected in the HSV-transformed or tumour cell lines. The experiments involved (1) co-cultivation of the transformed or tumour cells with human embryonic fibroblasts (HEF), and (2) plaque assay of extracts from sonically-disrupted transformed or tumour cells using HEF cell monolayers (L. S. Kucera & I. Edwards, unpublished data). The absence of detectable infectious HSV in the transformed or tumour cells was not surprising since the transforming virus was rendered ‘defective’ in its capacity to multiply by photoinactivation.

Assays for C-type retroviruses in our HSV-transformed rat cells were consistently negative after (1) 3H-uridine labelling of the transformed or tumour cells pre-treated or not treated...
with iododeoxyuridine (Panem et al. 1975), and examining cell extracts for particles banding in sucrose density gradients, or (2) examination by electron microscopy. However, these data do not exclude the possible presence or role of C-type viruses in HSV-oncogenesis. Munk & Darai (1975) detected C-type viruses in some HSV-transformed rat cells, however, these viruses were non-transforming in rat cells. The same authors (Munk & Darai, 1975) reported that other rat cells transformed with HSV under identical conditions were free of C-type viruses by all available detection techniques, yet these cells were oncogenic in rats.

In conclusion, results of our investigation are consistent with the idea that transformation by HSV may involve 'defective' viruses which have lost their capacity for replicating new infectious virus but retain their oncogenic capacity.

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