Br. J. exp. Path. (1976) **57**, 361

TRANSFORMATION OF PRIMARY HAMSTER EMBRYO FIBROBLASTS BY TYPE 2 SIMPLEX VIRUS: EVIDENCE FOR A " HIT AND RUN " MECHANISM

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Received for publication January 12, 1976

Summary.—The phenomenon of cell transformation by type 2 herpes simplex virus has been investigated. Primary hamster embryo fibroblasts were exposed to type 2 herpes virus under conditions which would restrict or inhibit the lytic events of virus–cell interaction. Cell lines were established by single-cell cloning.

There was evidence of altered cell morphology with altered biological activity in terms of longevity and oncogenicity; there was, however, no evidence of virus specific antigen or incorporation of viral nucleic acid into the host cell genome. Virus specific antigen was only detected in the early passages of an uncloned transformed cell line. We are thus unable to confirm previous studies (*vide supra*) and are obliged to propose a " hit and run " model for *in vitro* cell transformation by type 2 herpes simplex virus.

HERPES simplex virus, type 2, has been implicated in the aetiology of carcinoma of the uterine cervix. The association was first proposed by Naib, Nahmias and Josey (1966), who indicated that patients with a past or present history of herpes genitalis had an increased incidence of pre-invasive and invasive cervical carcinoma. This association has been in general confirmed by sero-epidemiological surveys, in which patients with pre-invasive or invasive cervical carcinoma have been shown to have an increased prevalence or titre or serum antibody to type 2 herpes simplex virus (Rawls *et al.*, 1968; Nahmias *et al.*, 1970; Royston and Aurelian, 1970; Skinner, Thouless and Jordan, 1971; Adam *et al.*, 1974).

Evidence of association does not, of course, indicate a cause-and-effect relationship and, on the assumption that the phenomenon of virus-induced cell transformation is analogous in at least certain aspects to the phenomenon of virus-induced cancerogenesis *in vivo*, a number of studies have therefore attempted to demonstrate that herpes simplex virus, or the virus DNA, can induce *in vitro* " transformation " of various cell types (Duff and Rapp, 1971*a*; Kutinova, Vonka and Broucek, 1971; Darai and Munk, 1973; Garfinkle and McAuslan, 1972; McNab, 1974; Takahashi and Yamanishi, 1974; Wilkie *et al.*, 1974). These studies in general suggest a model which is similar if not identical to " classical " accounts of papova- or adenovirus cell transformation, namely, altered cell and chromosomal morphology, oncogenicity and, of particular significance to this study, virus genome incorporation in the transformed cell lines (Black *et al.*, 1963; Habel, 1965; Huebner *et al.*, 1963).

In this study the phenomenon of cell transformation by type 2 herpes virus has been investigated. In one set of experiments, primary hamster embryo fibroblasts were exposed to u/v-inactivated type 2 herpes simplex virus and, in another set of experiments, the cells were exposed to live virus but the cultures incubated at the supra-optimal temperature of 40°. Cell lines were established by cloning from single cell colonies of transformed cells and characterized.

Our evidence suggests that type 2 herpes simplex virus is capable of *in vitro* cell transformation by a " hit and run " mechanism or, in more familiar terms, that the virus is capable, under certain experimental conditions, of acting as a cell mutagen. The evidence for this hypothesis and possible significance in relation to *in vivo* virus-induced cancerogenesis is presented.

MATERIALS AND METHODS

Cell culture.—BHK (C13) cells, a stable cell line derived from a single clone (MacPherson and Stoker, 1962) were used for routine virus propagation, antigen production and virus titrations. The cultures mentioned were in supplemented Eagle's medium (Vantsis and Wildy, 1962) containing 10% v/v calf serum and 10% v/v tryptose phosphate broth (ETC) under 5% carbon dioxide.

Primary hamster embryo fibroblasts.—These were prepared as described by Paul (1960). Ten-day-old hamster embryos were removed and the carcasses washed with phosphate-buffered saline. The embryos were then finely chopped up with scalpel and scissors, washed free of erythrocytes and digested with 0·05% trypsin at 37°. At 10-min intervals, 4-ml aliquots of the trypsin–cell suspension were removed, diluted 1/5 in freshly prepared ETC and cultivated under 5% carbon dioxide. Within 12 h there was evidence of cell seeding, at which time the medium was changed to remove cell debris and non-viable cells. Co-cultivation of the cells with BHK 21 or RK 13 cells did not rescue any detectable cytopathogenic agent.

All cell lines were routinely investigated for bacterial mycoplasmal or fungal contamination. For convenience, each cell line has been designated by a partially meaningful abbreviation; the first initials indicate whether the cell line is a transformed cell line (Tr) or a cell line established from an animal tumour (Tu); the second initial, H(40°) or u/v, indicates the mode of transformation of the cell line, or, in the case of a tumour cell line, the mode of transformation of the cell line initiating the tumour. The third initial indicates whether the cell line is a cloned (CL) or uncloned line (UNCL), *viz.*: (1) primary hamster embryo fibroblasts transformed at 40° cloned and uncloned—Tr H.CL and Tr H.UNCL respectively; (2) primary hamster embryo fibroblasts transformed with u/v-irradiated virus and cloned—Tr u/v. CL; (3) cloned tumour cell lines derived from animals inoculated with above transformed cell lines Tr H.CL and Tr u/v CL—Tu H.CL and Tu u/v respectively; (4) uninfected control primary hamster embryo fibroblasts—Prim. HEF; and (5) control baby hamster embryo fibroblast cells—BHK 21. C13 (MacPherson and Stoker, 1962).

Cell cloning.—The medium from a healthy semi-confluent cell sheet was decanted and the cell sheet washed twice with phosphate-buffered saline. The cells were removed by digestion for 10 min at 37° with trypsin–versene solution (0·05% trypsin, 1% ethylenediamine tetra acetate in de-ionized water) and the digestion process inhibited by addition of 4 vol of cold ETC medium. The cells were dispersed and thereafter dispensed, in serial dilutions, into 25-mm plastic Petri dishes. Single colonies of cells were usually apparent within 3–5 days; these were easily picked up under direct vision using a Pasteur pipette. This procedure was thrice repeated and a stock of cells cultivated.

Viruses.—Strain " 3345 ", a " prototype " type 2 strain of herpes simplex virus (Geder and Skinner, 1971; Plummer *et al.*, 1974), was used in the transformation experiments. Strain HFEM, a derivative of the Rockefeller strain HF (Wildy, 1955), was used as a " prototype " type 1 herpes simplex virus strain.

Inactivation of virus.—Virus was routinely irradiated at a dose rate of 1118 erg/mm^2/min. Following 8 min irradiation, less than 10,000-fold original infectivity remained. In our transformation experiments, cells were infected with virus irradiated to this survival.

Antisera.—Antigen for immunization of rabbits was prepared in rabbit kidney cells (RK 13 cell line; Beale, Christofinis and Furminger, 1963) according to the method of Watson *et al.* (1966). Rabbits were inoculated i.m. with 100 mg of freeze-dried formalin-inactivated antigen with Freund's incomplete adjuvant and then at monthly intervals with equivalent doses of " live " antigen for 5 doses. Thereafter, the rabbits were given booster immunizations at 3-monthly intervals. Rabbits were bled 14 days following each booster immunization.

Serological techniques

Immunofluorescence.—By seeding a 50-mm diameter Petri dish containing a plastic coverslip or coverslip fragments with approximately 10^6 transformed cells, a sparse cell sheet was obtained within 16 h (overnight) which was most convenient for immunofluorescence staining. Prior to fixation the cell sheet was washed 3 times with phosphate-buffered saline.

The indirect method using hyperimmune rabbit sera and sheep anti-rabbit globulin conjugated to fluorescein isothiocyanate was employed. The method of conjugation and removal of non-specific staining from conjugated antisera has been described by Ross, Watson and Wildy (1968). Prior to use the immune rabbit antisera were absorbed as follows: 1 ml

of the antiserum was added to 10^8 phosphate-buffered saline-washed BHK 21 cells in pellet, shaken at 37° for 1 h and then overnight at + 4°. These cells were removed by centrifugation and the supernatant serum added to 10^8 BHK 21 cells which had been washed, methanol-fixed, and then rewashed. The new serum cell suspension was shaken at 37° for 1 h and again overnight at +4°, and the methanol-fixed cells removed by centrifugation at 5000 rev/min for 20 min, and ultracentrifugation at 20,000 rev/min for 1 h.

Slide preparations were examined under the Zeiss photomicroscope using exciter-filters BG 3/4 and BG 12/4 and barrier filters 53, 50 and 44.

Complement fixation tests.—Antigen for complement fixation testing was prepared by ultrasonically disintegrating 10^7 washed transformed cells in 1 ml of complement fixation diluent. The suspension was then clarified by centrifugation at 5000 rev/min for 20 min. Complement, obtained in dried form, was used in a dose of 4 haemolytic units. Sheep red cells were sensitized overnight at + 4° using 1/800 dilution of horse anti-rabbit haemolytic serum. These reagents were obtained from Burroughs Wellcome Reagents. The cells were added to the reaction mixture at a concentration of 5×10^7/ml.

Absorption of antisera with transformed cells.—One ml of 1/40 dilution in saline of a hyperimmune type 1 or type 2 rabbit antiserum was added to 5×10^7 saline-washed cells in a pellet and shaken for 1 h at 37°, and then overnight at + 4°. The cells were then removed by centrifuging at 2000 rev/min for 15 min and the supernatant-absorbed serum added to a fresh 5×10^7 cells which were disrupted ultrasonically in the serum, shaken for 1 h at 37° and, again, overnight at + 4°. Cell debris was then removed by ultracentrifugation at 100,000 *g* for 1 h at 4°. To control for non-specific removal of antibody from such a dilute serum, parallel absorptions were always carried out on a separate aliquot using uninfected BHK 21 cells and uninfected primary hamster kidney cells.

DNA hybridization techniques.—These tests were kindly carried out by Dr A. Minson and Dr G. K. Darby in the Department of Virology, University of Birmingham. In brief, the cells were examined for the presence of herpes DNA using ^{125}I-labelled type 2 herpes DNA of high specific activity ($\sim 10^7$ cpm/μg) as a probe. The renaturation kinetics of the probe DNA were determined in the presence of transformed cell DNA, and these were compared to the kinetics in the presence of BHK DNA and also in the presence of BHK DNA and type 1 herpes DNA equivalent to 1 genome/cell.

Chromosome analysis.—In brief, cell cultures were treated with 0·02% colchicine for 1 h at 37°, the cells removed by trypsinization, pelleted by gentle centrifugation, and resuspended for 20 min at 37° in hypotonic potassium chloride (0·075M) which served to swell the cells. Following repelleting, the cells were carefully resuspended (to avoid clumping) in 1 : 3 glacial acetic acid–methanol fixative.

RESULTS

Transformation by type 2 herpes virus at 40°

Primary hamster embryo fibroblasts, which had been passaged 3 times since establishment of the cell line, were used. Replicate aliquots containing 3×10^7 cells were absorbed in suspension for 60 min with 60 and 6 PFUs/cell of type 2 virus strain 3345. The cell suspensions were incubated at 10^6 cells per Petri dish at 40° with a parallel set of cultures at 37° for control purposes. As the particle–infectivity ratio of this virus preparation was approximately 1000, an input multiplicity of 60 PFU/cell ensured, from Poissonian considerations, that every cell was " hit " by at least one infective virus particle and at the lower multiplicity of 6 PFU/cell by at least one physical herpes virus particle.

In cultures infected with 60 PFU/cell, following 6 h incubation, there was, at both temperatures, a 100% cytopathic effect with every cell positive (by immunofluorescence) for herpes virus antigen. By 16 h, the cell sheet was clearly destroyed, there being only occasional cells still attached to the Petri dish.

In cultures infected with 6 PFU per cell, however, following 6 h incubation, occasional spindle cells were apparent at both temperatures. By 16 h, cultures at 37° were completely destroyed by virus infection. On this account, attention was entirely focused on any remaining cultures where there was evidence of viable cells, namely, the cultures infected with 6 PFU/cell which had been incubated at 40°. Similar repeat procedures confirmed these findings, namely, that multiplicities of infection greater than 4–6 PFU/cell were lethal to primary hamster embryo fibroblast cultures even at the supra-optimal temperature of 40°.

In the course of the next 3 or 4 days it

was apparent that these occasional cells were multiplying and, after 10 days, foci of " piled-up " cells were visible to the naked eye. There were respectively 10, 9, 6, 6, 0 and 0 foci of cells on 6 replicate Petri dishes. These colonies consisted of irregularly arranged, somewhat epithelioid, cells. The medium was changed at 3-day intervals and on each day the supernatant medium titrated for infectious virus. It was apparent that after 5 days' incubation at 40° there was no infectious virus in the supernatant fluid and, on this account, the cultures were transferred to 37°. Thereafter, there was no cytopathic effect and no infectious virus production.

Transformation by type 2 herpes virus inactivated by u/v irradiation

Replicate aliquots of 3×10^7 thrice-passaged primary hamster embryo fibroblasts were exposed to 3×10^9 and 3×10^8 " pre-inactivation " PFU/cell of this virus preparation; at these multiplicities, every cell would be " hit " by at least 1 pre-inactivation infective virus particle. Following 1 h absorption at 37°, 10^6 cells were dispensed into Petri dishes and cultivated in routine fashion at 37°.

Following 6 h incubation, the cultures infected with 100 pre-inactivation PFU/cell were entirely " rounded-up " with no evidence of cell spindling or cell seeding. In cultures infected with 10 pre-inactivation PFU/cell, there was also a gross cytopathic effect but very occasional spindle cells, varying from 0–10/10^5 cultured cells, were evident. Immunofluorescent microscopy revealed that 296 of 300 cells were positive for herpes virus antigens. The cultures were washed 3 times with growth medium and, to inhibit the spread of infective virus particles, were recultured in 5 ml growth medium containing 20% carboxy-methyl cellulose (ETC CMC). After a further 5 days' incubation there was evidence of growth and division of fibroblastic cells in the culture, accompanied in other areas by obvious cytopathic effect of herpes virus infection, the supernatant medium containing on average 10^6 PFU virus/ml. At this juncture, there were no distinct foci or colonies of transformed cells. In an attempt to remove infective virus from these cultures, the cell sheets were washed thoroughly with saline and the cells removed from the Petri dishes by tryspin–versene. They were replated at varying cell concentrations per Petri dish under ETC–CMC medium. After a further 5 days' incubation, around 10–20 colonies were apparent on Petri dishes seeded with 10^5 cells and these were " picked " and replated in similar fashion. It was necessary to repeat this procedure 2 further times before a virus-free culture was obtained. Virus titres in this supernatant medium from these 3 successive " platings out " were on average 4×10^4/ml, 6×10^3/ml and 7×10^0/ml respectively. The cells, now at their fourth passage since " transformation ", appeared entirely fibroblastic. Six clones were independently derived from single cell clonings and stocks of cells grown up for further characterization.

In the course of this study, 7 independent transformation experiments (3 using u/v-irradiated virus and 4 at supra-optimal temperature) have been undertaken. The procedure and experimental observations were essentially as described, permanent cell lines being obtained on each occasion.

Control cell lines

Uninfected control primary hamster embryo fibroblasts.—As far as possible, uninfected hamster embryo cells were passaged in similar fashion. However, attempts to clone these cells from single cells were unsuccessful and in all of 8 primary cultures cell replication had all but ceased by the tenth passage. This was more apparent with the control uninfected cells at 40° which had ceased to replicate by only their fifth passage. On this account, control experiments were carried out on uninfected hamster embryo cells which had reached their sixth passage level at 37°.

Uninfected continuous hamster cell line.

—Baby hamster kidney cells, a continuous cell line (BHK 21 C13), were also included for control purposes.

Properties of transformed and tumour cell lines

Morphology.—Both " H " and " u/v " transformed cell lines usually grow in an irregular pattern forming " piled-up " foci if seeded at low density (Fig. 1). With passage, however, the cells formed a more regular pattern of growth with parallel alignment of cells until, by the twentieth passage, the pattern of growth was indistinguishable from that of the uninfected primary hamster embryo fibroblasts or the BHK 21 cell line (Fig. 2).

The individual cell morphology of both the H and u/v cell lines showed features not apparent in the control primary hamster cell line or in the BHK 21 cell line.

(1) Confluent cultures of both H and u/v cells regularly yielded lower counts of cells than confluent cultures of BHK 21 cells; in addition, the individual H and u/v cells, when examined in a counting chamber, for example, appeared to be considerably larger than BHK 21 cells.

On this account, 100 cells of each cell line, suspended in isotonic saline in a counting chamber, were measured with a calibrated micrometer eye-piece. The transformed cell lines Tr u/v (CL) Tr H(CL) had mean average cell diameters of 0·08 mm ($\pm$ 0·005 mm) and 0·092 mm ($\pm$ 0·008 mm) respectively, while the control BHK 21 cells had a significantly lower mean average cell diameter of 0·051 mm ($\pm$ 0·001 mm) ($P < 0{\cdot}01$). It is interesting that the scatter of diameter values is also significantly higher for both transformed cell lines—a consequence of the increased pleomorphism of these cells.

(2) The transformed cell line Tr u/v (CL) contained a varying proportion of multinucleate giant-cells; in some cultures 2–3% of the cells were multinucleate with as many as 7 nuclei in certain multinucleate cells. The significance of these cells remains obscure; their prevalence could not be increased by varying the conditions of culture and, to date, attempts to establish a cell line from a " single " multinucleate cell have been unsuccessful. The cells did not appear to show any typical features of herpes virus infection and did not contain virus specific antigen. No infectious agents were rescued from these cultures.

Cell lines derived from tumours in hamsters also show considerable pleomorphism with increased nuclear–cytoplasmic ratio. Multinucleate cells were never visualized.

Preliminary examination of 100 cells from the eleventh passage of the transformed cell line Tr u/v CL indicated that approximately 80% of the cells were diploid, 15% were tetraploid and the remaining 5% of higher ploidy. Control primary or BHK 21 cells contained a similar but slightly higher proportion of diploid cells—approximately 90%. It was noted, however, that the transformed cells contained a high frequency of dicentric chromosomes and, of possibly great interest, a minute marker fragment. These features were not apparent in either control cell line (Harnden, D. G., personal communication).

Biological properties

Efficiency of plating.—The values for 4 independently determined plating efficiencies are recorded for each cell line in Table I. There was no significant difference in the mean plating efficiency of the " continuous " cell lines, neither the transformed nor tumour cell lines having a significantly higher or lower value than the BHK 21 control cell line. The control primary hamster cells had extremely low plating efficiencies. There was considerable scatter within replicate platings for a given cell line; this was thought to be related to the inaccuracies of cell counting compounded by the subsequent dilution steps necessary in obtaining a final 10^2 and 10^3 cells per Petri dish.

Cloning efficiency in soft agar.—Colony formation was investigated in 0·5% agar using a 0·3% agar overlay. Three-

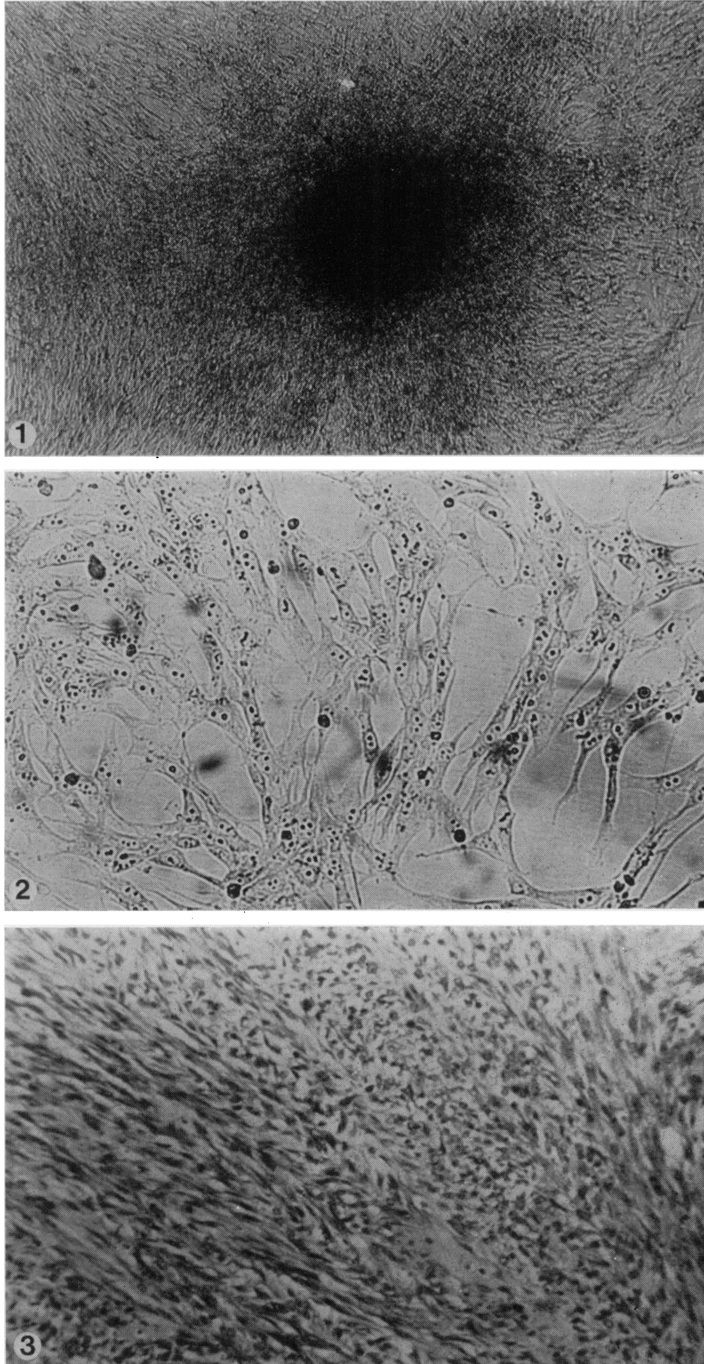

FIG. 1.—Transformed cell line Tr u/v (CL) at 3rd passage; typical piled-up "focus" of cells. Unstained preparation. × 24.
FIG. 2.—Control hamster kidney cell line (BHK 21). Unstained preparation. × 213.
FIG. 3.—Histological section of tumour; typical feature of fibrosarcoma. × 106.

TABLE I.—*Plating Efficiency; Transformed, Tumour and Control Cell Lines*

Cell line	Passage level	Plating efficiencies (%) colonies formed/number of cells seeded (× 100)				Mean plating efficiency %
Transformed cell lines						
Tr H (UNCL)	9	21	7	5	5	9·5
Tr H (CL)	16	14	4	12	12	10·5
Tr U/V (CL)	9	0·9	0·2	13	3	4·25
Tumour cell lines						
Tu H (UNCL)	8	ND				
Tu H (CL)	4	6	48	17	19	22·5
Tu U/V (CL)	4	0·6	11	18	14	10·75
Control cell lines						
Primary HEF	6	0·01	0·01	0·01	0·01	0·01
BHK 21	>10	9	3	8	4	6·0

TABLE II.—*Oncogenicity Titrations; Transformed, Tumour and Control Cell Lines; Live and Inactivated Type 2 Herpes Simplex Virus*

		Tr H (CL) Week of inspection							*Tr u/v (CL)* Week of inspection					
Dose	Passage level	1	2	3	4	5	6	Passage level	1	2	3	4	5	6
10^7	14	2/7*	6/7	7/7	—	—	—	11	6/6	—	—	—	—	—
10^6	14	0/10	0/10	4/10	10/10	—	—	11	7/7	—	—	—	—	—
10^5	16	0/7	0/7	1/7	1/7	—	—	11	3/5	5/5	—	—	—	—
10^4	16	0/5	0/5	0/5	0/5	0/5	0/5	14	4/6	6/6	—	—	—	—
10^3								16	1/4	1/4	3/4	3/4	3/4	3/4
10^2								16	0/8	1/8	3/8	5/8	—	—
10^1								16	0/8	0/8	0/8	2/8	2/8	2/8
Tumour cell lines														
		Tu H (CL)							*Tu u/v (CL)*					
10^3	6	3/6	6/6	—	—	—	—	6	2/6	4/6	6/6	—	—	—
10^2	6		Litters died					6	0/4	2/4	3/4	3/4	3/4	3/4
10^1	6		Litters died					6	0/7	0/7	0/7	1/7	1/7	1/7
Control cell lines														
		BHK-21							Prim. H.E.F.					
10^7		2/9	5/9	9/9	—	—	—	6	0/10	0/10	0/10	0/10	0/10	0/10
10^6		1/6	2/6	4/6	4/6	4/6	4/6	6	0/4	0/4	0/4	0/4	0/4	0/4
10^5		0/8	3/8	3/8	4/8	4/8	4/8							
10^4		0/0	0/0	0/0	1/6	1/6	1/6							
Type 2 herpes virus														
		Infectious virus							*Non-infective virvs*†					
10^3 PFUs		—	—	—	—	—		(8/8 fatality)	(1) u/v irradicated (10 min at dose rate of 1118 erg/mm²)					0/7
10^2 PFUs		0/4	0/4	0/4	0/4	0/4		(5/9 fatality)	(2) heat inactivated (60 min at 60°)					0/4
10^1 PFUs		0/6	0/6	0/6	0/6	0/6		(0/6 fatality)	(3) trypsin digested (0·2% trypsin for 30 min at 37°)					0/10

* Numerator—number of animals with tumour. Denominator—number of animals inoculated.
† Virus dose of 10^6 " erstwhile " and 10 " surviving " PFUs per newborn animal.

dimensional colonies of cells were only obtained with the control BHK 21 cell line. The cloning efficiency of these cells was low, varying from 2–24 colonies/10^5 BHK 21 cells.

Growth rate.—25-mm plastic Petri dishes were needed with 2×10^5 cells and cultured as previously described. At daily intervals, the cells from 4 replicate Petri dishes were removed by trypsin–

versene and counted. Medium was replenished after 3 days' incubation. The average daily cell counts are shown in Fig. 3.

There was little difference in the initial replication rate; however, the maximum count attained for each cell line was approximately 10^6/Petri dish for both transformed cell lines and over 5×10^6/ Petri dish for the tumour cell lines and BHK 21 cells. At these cell densities, the cells formed confluent monolayers. These observations are consistent with the greater size of the 2 transformed cell lines (*vide supra*).

Oncogenicity.—The results of oncogenic titrations in newborn hamsters for transformed tumour and control cell lines and for live and inactivated type 2 herpes simplex virus are shown in Table II. The data are imperfect on account of the unpredictability of littering times which would thus require a very large number of pregnant hamsters to permit, for example, simultaneous inoculation of perhaps 7 different dosages of a given cell line at the same passage level.

The transformed cell lines were clearly more oncogenic than the control uninfected primary cell line in which inocula of 10^7 have, to date (9 months), failed to produce a single tumour. This was particularly so for the cell line transformed with u/v-inactivated type 2 herpes Tr u/v (CL), which was approximately 1000-fold more oncogenic than the control BHK 21 cell line. Although the data are scant concerning the tumour cell lines, their oncogenicity appeared to be similar to the transformed cell line Tr u/v (CL). It was interesting that on not one occasion did a tumour develop more than 28 days after inoculation. Tumours were not obtained following inoculation of live or inactivated virus.

The tumours were initially well-encapsulated and seemed not to metastasize. On section, there were large areas of haemorrhage and necrosis and examination revealed the typical features of fibrosarcomata (Fig. 4).

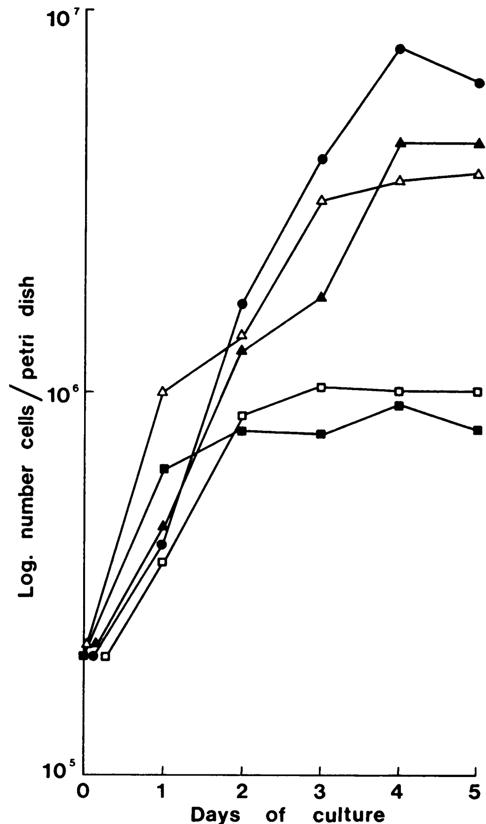

FIG. 4.—Growth rate of transformed, tumour and control cell lines. □——□ Cell line Tr u/v Cl; ■——■ cell line Tr H CL; △——△ cell line Tu u/v CL; ▲——▲ cell line Tu H CL; ●——● cell line BHK 21.

Evidence of virus genome in transformed cell lines

Rescue of infective herpes virus.—Co-cultivation of the transformed, tumour and control cell lines with susceptible lines of BHK 21 cells and treatment of the cell cultures by addition of 5 μg/100 ml of iodoxyuridine to the culture medium did not induce infective virus formation, assembly of herpes or other virus particles or virus-specific intracellular or cell membrane antigen formation.

Interference with virus plaquing and virus replication.—Type 1 strain, HF, and type 2 strain, " 3345 ", were simultaneously titrated in each cell line. There

was no difference in the titres obtained for either virus.

The replication of these 2 strains in transformed cell line Tr u/v (CL), its induced tumour cell line, Tu u/v (CL), and in control BHK 21 cells was investigated. The cells were infected with 10 BHK PFU/cell and residual virus removed by incubation of the cells for 5 min with trypsin–versene. There was replication of both virus types in all 3 cell lines.

Virus-specific antigens

(a) *Immunofluorescence.*—The results have been expressed as the percentage of cells showing unequivocal evidence of fluorescent antigen. Tests were always carried out under code, the investigator having no knowledge of whether test or control preparations were under examination. It is apparent that in transformed or tumour cell lines, which had been rigorously cloned, there was no evidence of fluorescent antigen formation (Table III). The highest and only significant proportion of antigen-containing cells was found in the uncloned cell line TR H UNCL; however, it is clear that the proportion of antigen-containing cells in this cell line progressively decreased with cell passage until by the fourteenth passage there was no detectable antigen formation. The fluorescence consisted of coarse cytoplasmic granules and occurred in rather "rounded" cells in which the other details of cell morphology were usually obscured by the diffuse pattern of fluorescence. The possibility that these cells were "dead" or "dying" cells which were non-specifically binding globulin molecules was negated by the very low background fluorescence ($< 0{\cdot}1\%$) obtained in replicate preparations treated with non-immune rabbit serum, and with hyperimmune rabbit serum to an unrelated set of antigens (anti-mycoplasma serum). Immunofluorescent tests on whole unfixed cell preparations failed to reveal virus-specific cell surface antigens in any of the uncloned cell lines.

(b) *Complement fixation.*—The results agree with the immunofluorescent findings; only uncloned transformed cell line Tr H UNCL extract reacted to a significant titre with hyperimmune anti-viral serum (Table III). The result of comparative "chess-board" titrations with this cell line suggested that the reacting virus antigens were type 2 (Table IV). This is consistent with the "type" of the transforming virus.

TABLE III.—*Virus-specific Antigens in Transformed, Tumour and Control Cell Lines*

Cell line	Immunofluorescence Proportion of positive cells (%) (200 cells counted)	Complement fixation with hyperimmune anti-type 2 sera	Absorption of neutralizing antibody from type 2 hyperimmune sera k-value v	
			Type 1 virus	Type 2 virus
Transformed cell lines				
Tr H (UNCL)				
*0 passage	99	ND	2·81	1·33
3rd passage	6·5	1/128†	2·66	1·44
6th passage	5·5	1/32	3·47	2·71
10th passage	<0·5	1/4	3·31	2·60
14th passage	<0·5	1/4	3·41	2·98
16th passage	<0·5	1/4		
Tr H (CL)	<0·5	1/4	3·66	2·88
Tr u/v (CL)	<0·5	1/4	3·40	2·94
Tumour cell lines				
Tu H (Cl)	<0·5	1/4	3·22	2·36
Tu u/v (CL)	<0·5	1/4	3·19	2·06
Control cell lines				
Prim. HEF	<0·5	1/4	ND	ND
BHK-21	<0·5	1/4	3·52	2·24

* 16 h following infection of culture with herpes virus.
† End-point dilution of antiserum.

TABLE IV.—*Type Specificity of Virus Specific Antigen in Uncloned Transformed Cell Line Tr H UNCL*

	Antiserum to type 1 infected cells	Antiserum to type 2 infected cells	Ratio of titres
Cell extract from cell line Tr H UNCL	1/32*	1/128	4
Type 1 herpes antigen	1/1024	1/512	0·5
Type 2 herpes antigen	1/64	1/512	8

* End-point dilution of antiserum.

(c) *Absorption of neutralizing antibody.*—The effect of absorption of a dilute hyperimmune type 2 antiserum with both whole cell and sonicated cell extract is shown in Table III. There was a significant reduction of both type 1 and type 2 neutralizing activity only when the antiserum was absorbed with early-passage cell extracts of uncloned transformed cell line Tr H UNCL. Later passage levels of this cell line did not, however, remove any neutralizing antibody from this antiserum.

(d) *Virus-specified thymidine kinase activity.*—These tests were kindly carried out by Dr M. E. Thouless. There was no evidence of increased enzyme activity over host enzyme activity in any of the transformed or tumour cell lines.

(e) *Immune reactivity in serum of adult tumour-bearing animals.*—For the particular purpose of investigating the immune reactivity in the sera of tumour-bearing animals, adult hamsters were inoculated with 10^6 transformed cells and were bled whenever the induced tumour became macroscopically apparent.

(f) (1) *Neutralizing antibody.*—To obtain maximum sensitivity in this test, the sera were tested at a dilution of 1/4 in the presence of complement (8 haemolytic units per reaction mixture) and reacted at 37°. In agreement with the above findings, significant neutralizing activity was only found in the sera of tumour-bearing animals in which the tumour had been derived from inoculation of the uncloned transformed cell line Tr H UNCL (Table V). There was no apparent correlation between the rapidity of tumour induction, the rate of tumour growth or the tumour mass at cardio-puncture with the occurrence or titre of neutralizing antibody. In addition, adult hamsters which were transplanted with tumour fragments from tumour-bearing animals (1) to (7) (Table V) did not develop serum neutralizing antibody. Low levels of antibody were also found in 2 animals inoculated with this cell line, Tr H (UNCL), who had not developed tumours. There was no serum neutralizing antibody in animals bearing tumours induced by either cloned transformed cell line.

(2) *Complement-fixing antibody.*—Of the sera tested in Table V it was curious that only sera from tumour-bearing animals Nos. 3 and 5 contained a recordable titre of complement-fixing antibody. " Chess-board " titrations using these sera against both type 1 and type 2 infected BHK cell antigen, and against antigen prepared from equivalent cell numbers of the cloned and uncloned transformed cells and control uninfected BHK 21 cell antigen, are shown in Table VI. Comparison of the results with the behaviour of immune antisera of known immunogenic type suggests the presence of type 2 antibody which is again consistent with the type of the original transformed virus. It was interesting that only antigen from the uncloned cell lines Tr H (UNCL) fixed complement with one of the antisera; no reactivity was noted with antigen from the cloned cell lines or from the BHK 21 cell line.

Virus DNA.—A preliminary investigation of the cloned highly oncogenic cell line Tr u/v CL was investigated by DNA–DNA hybridization using iodine-labelled type 2 herpes DNA. There was less than 1 genome of herpes DNA per transformed cell, the latter representing the limit of sensitivity of the test system (Minson, A. and Darby, G. K., personal communication).

TABLE V.—*Neutralizing Antibody in Sera of Tumour-bearing Hamsters*

Cell line (passage number in brackets)	Log decrease in infectivity following incubation with antiserum for 1 h at 37°		Ratio Type 1/Type 2
	Type 1 herpes (strain " HF ")	Type 2 herpes (strain " 3345 ")	
Tr H (UNCL) (7)			
Hamsters developing tumours			
Hamster (1)	0·73	0·34	2·1
Hamster (2)	0·81	0·07	1·6
Hamster (3)	0·16	0·18	0·88
Hamster (4)	0·06	0·14	0·43
Hamster (5)	0·09	0·00	—
Hamster (6)	0·00	0·00	—
Hamster (7)	0·00	0·00	—
Hamsters transplanted with tumours from each of above (7 hamsters tested)	0·00	0·00	—
Hamsters not developing tumours			
Hamster A	0·09	0·04	2·3
Hamster B	0·14	0·00	—
Hamster C	0·00	0·00	—
Hamster D	0·00	0·00	—
Hamster E	0·00	0·00	—
Hamster F	0·00	0·00	—
Hamster G	0·00	0·00	—
Hamster H	0·00	0·00	—
Tr H (CL) (15) (12 hamsters tested)	0·0	0·0	—
Tr u/v (CL) (16) (18 hamsters tested)	0·0	0·0	—

TABLE VI.—*Complement Fixation by Sera from Tumour-bearing Animals*

Sera	Antigen				
	Type 1 herpes virus	Type 2 herpes virus	Tr H (UNCL)	Tr H (CL)	BHK-21
Tumour-bearing hamster No. 3	1/32*	1/64	1/16	1/4	1/4
Tumour-bearing hamster No. 5	1/8	1/8	1/4	1/4	1/4
Antiserum to type 1 infected cells	1/1024	1/128	1/16	1/4	1/4
Antiserum to type 2 infected cells	1/512	1/512	1/16	1/4	1/4

* End point dilution of antiserum.

DISCUSSION

In this study, the role of type 2 herpes virus in cervical cancerogenesis has been investigated at a cellular level. The experimental results will be considered under 4 headings.

1. *Evidence of cell transformation*

There was clear evidence of cell transformation. In contrast to the control non-oncogenic primary hamster embryo cell, the " H "- and " u/v "-transformed cells formed continuous cell lines which were oncogenic in both newborn and adult hamsters. While it has been unequivocally demonstrated that the oncogenicity of a given cell line increases *in vitro* passage (Jarret and MacPherson, 1968), it seems unlikely that our results, where the transformed cell lines were passaged perhaps only 6 times more than control cell lines (Table II), can be thus explained.

Evidence of morphological cell trans-

formation was less clear-cut. While the transformed cell lines did not form piled-up foci of randomly orientated cells beyond their fifteenth passage, the individual transformed cells were larger and more pleomorphic, with a varying proportion of multinucleate cells in the " u/v "-transformed cell line. These multinucleate cells showed no evidence of herpes virus antigen and their significance remains obscure. There was preliminary evidence of a marker chromosome in transformed cell line Tr " u/v " CL. It is clearly necessary to establish the clonal proliferation of this marker by further experimentation.

2. *Evidence implicating type 2 herpes virus in cell transformation*

In terms of physical particles this multiplicity of infection (6000/cell) ensured that every cell was " hit " by at least 2, if not more, virus particles. Thus the continuous cell lines could have been derived from:

(a) Cells which were " hit " by infective particles in which, for as yet undetermined reasons, the lytic cycle did not continue and instead cell transformation occurred.

(b) Cells which were " hit " by non-infective virus particles; this latter situation generates 2 possibilities:

(i) The cell becomes transformed by one or more " non-infective particles " or a combination of " non-infective " and infective particles. That this can occur has not been unequivocably proven although the study of Latarjet, Carmer and Montagnier (1967) does suggest that u/v-irradiated SV40 particles may retain their oncogenicity while losing their infectivity.

(ii) The cell remains unaltered by exposure to virus particles, *i.e.* it is biologically an uninfected cell. In this instance, one must speculate that the acquisition of new properties was a result of transformation by an undefined agent in the test system not present in the control system—this is unlikely in view of the adoption of mock conditions of transformation in the control system—or, secondly, was a result of " spontaneous " transformation of uninfected cells. This phenomenon, reported as early as 1941 for rat cells (Gey, 1941), mouse cells (Earle, 1943) and for human amnion cells (Regan and Smith, 1965) is, however, a rather rare event with hamster cells (Tsuda, 1965; Diamond, 1967).

The most likely hypothesis, therefore, is that transformation was effected by type 2 herpes virus. The alternative—that our experimental procedure selected a cell, which, although exposed to at least 2 virus particles, remained uninfected and " unaffected " by the virus, but had nevertheless acquired new properties as a result of spontaneous transformation by some other undetermined agent(s)—must be adjudged considerably less likely.

3. *Comparison with other virus-cell transformation systems*

While the findings present certain features which occur in other virus-cell transformations, there is little overall parallel with the " classical " papova- or adenovirus system, with the RNA leucovirus systems, or indeed with other reports of type 2 herpes virus induced cell transformations.

The establishment of a continuous cell line with altered growth characteristics from a primary or non-viable cell line finds a parallel in herpes simplex and EB virus transformation (Duff and Rapp, 1971*b*; Henle *et al.*, 1967; Pope, Horne and Scott, 1969), in SV40 and polyoma virus (MacPherson and Stoker, 1962) and Rous sarcoma virus cell transformation (Manaker and Groupe, 1956).

The oncogenicity of our transformed cell lines (*vide supra*) is a general feature of virus-induced cell transformation. On the other hand, inoculation of live or

inactivated type 2 herpes virus did not initiate tumour formation. This may not be entirely unexpected, the extreme susceptibility of newborn hamsters to herpes virus (in our system the LD_{50} was approximately 10^2 BHK PFU) permitting inoculation of only a small number of infective particles. Secondly, the curious ease with which adenoviruses, papovaviruses and RNA leucosis viruses induce tumours in rodent species has led to the assumption that rodent species provide a good " general indicator system " for viral oncogenesis. For herpes viruses, where herpes virus saimiri and aeteles will induce tumours only in certain primate species (Ablashi *et al.*, 1971), this assumption may well be in error.

Our failure to rescue virus infectivity is in agreement with Duff and Rapp's observations with type 2 herpes simplex virus transformation. In this latter study, however, the authors did report evidence of virus antigen in the transformed cells although their failure to establish cloned cell lines and to specify the passage level of the antigen-containing cells under investigation suggests the possibility that their " transformed " cell lines may represent chronically infected cultures with a low, possibly very low, proportion of infected cells. These considerations are consistent with our own study, where virus antigens were detected only in the early passage levels of uncloned cell lines and immune reactivity was detectable only in the tumour-bearing hamsters inoculated with these early uncloned cells, but never in hamsters transplanted with tumours from antibody-positive animals. There may of course be incorporation of viral DNA without virus antigen formation (*e.g.* Raji cell line—Zur-Hausen *et al.*, 1970). However, our preliminary investigations revealed less than 1 genome per cell of virus DNA in our highly oncogenic cell line (Tr u/v CL).

Thus, in view of our repeated failure to demonstrate viral " footprints " in any of our cloned transformed cell lines, we are obliged to suggest a " hit and run " mechanism for herpes simplex virus cell transformation. This is an " uncomfortable " hypothesis, partly as there is little parallel for such a phenomenon in contemporary virology and partly because of the inherent difficulty in experimentally confirming an aetiologic role for the virus, which, having done the deed, is no longer present to be identified in any shape or form. One is thus compelled to adopt a semi-epidemiological approach, *i.e.* establish a high score of successful cell transformations with the virus in comparison to a significantly lower score of unsuccessful transformations under appropriate control conditions.

4. *Significance of results in relation to cervical cancerogenesis*

The general relevance of virus-induced *in vitro* cell transformation to human cancerogenesis remains unproven and evidence relating herpes simplex virus cell transformation to human cancerogenesis is fragmentary. While Royston and Aurelian (1970) claimed to have identified herpes virus antigens in dyskaryotic cervical cells and Frenkel *et al.* (1972) have reported DNA fragments in cervical tumour cells, the absence of any subsequent reports confirming these exciting observations is worrying.

In our system, as evidence of " classical " SV40-like cell transformation was not obtained, we must therefore propose that, if type 2 herpes virus is indeed related to cervical carcinoma, the virus transform cervical epithelial cells in the manner suggested by our transformation experiments. Confirmation of this postulate, where the proposed aetiological agent is " conspicuous by its absence ", is even more impenetrable than in the *in vitro* transformation system. However, with reference to human cancerogenesis, the hypothesis has certain recommendations.

In the first place, this situation is the rule rather than the exception for human tumours of proven aetiology; one would be hard-pressed, for example, to find

intracellular " footprints " providing unequivocal evidence that a given human tumour had been induced by X-irradiation or carcinogenic chemicals, and yet the oncogenicity of these agents is beyond doubt. In other words, a carcinogenic agent may initiate tumour formation and thereafter disappear. This concept will find little support among present-day tumour virologists whose enthusiasm, generated by the spectacular results with SV40 and adenovirus, may have transcended proper reserve over the general applicability of their findings to other virus transformation or tumour systems.

Secondly, the absence of virus antigen from our transformed cells and the apparent absence of transplantation antigens from other herpes simplex virus transformed cell lines (Duff, Doller and Rapp, 1973) will presumably discourage immune rejection of herpes virus-induced tumours, given of course that these findings obtain *in vivo*. This may be relevant as over 70% of the population has had previous exposure to type 1 herpes virus by adolescence (Nahmias *et al.*, 1970).

It is tempting, therefore, to accommodate our experimental results and these various observations into a unified hypothesis, namely, that following infection of human cervical epithelial cells with type 2 herpes virus, a low, possibly very low, proportion of epithelial cells are transformed in the manner described in this study. These cells have a selection advantage on account of their increased oncogenicity and, being antigen-free, are not susceptible to immune destruction. In other words, the body is presented with the equivalent of our uncloned transformed cell line and unwittingly " clones " the tumourogenic antigen-free transformed cells. It is emphasized that with the presently available data these suggestions are necessarily speculative.

I am grateful to Drs A. Minson and G. K. Darby for carrying out nucleic acid hybridization studies, to Dr M. E. Thouless for thymidine kinase assays, to Professor D. Harnden, Department of Cancer Studies, University of Birmingham, for chromosome analyses, and to Professor P. Wildy and Dr A. Buchan for much useful advice and criticism. Mrs S. Trueman and Mrs J. Edwards provided invaluable technical assistance.

REFERENCES

Aaronson, S. A. & Todaro, G. J. (1968) SV40 Antigen Induction and Transformation in Human Fibroblast Cell Strains. *Virology*, **36**, 254.

Ablashi, D. V., Loeb, W. F., Valerio, M. G., Adamson, R. H., Armstrong, G. R., Bennett, D. G. & Heine, V. (1971) Malignant Lymphoma with Lymphocytic Leukemia in Owl Monkeys Induced by Herpesvirus Saimiri. *J. natn Cancer Inst.*, **47**, 837.

Adam, E., Kaufman, R. H., Melnick, J. L., Levy, A. H. & Rawls, W. E. (1973) Seroepidemiologic Studies of Herpes Virus Type 2 and Carcinoma of the Cervix IV. *Am. J. Epidem.* **98**, 77.

Aurelian, L., Strandberg, J. D., Melendez, L. V. & Johnson, L. A. (1971) Herpesvirus Type 2 Isolated from Cervical Tumour Cells Grown in Tissue Culture. *Science*, **174**, 704.

Beale, A. J., Christofinis, G. C. & Furminger, I. G. S. (1963) Rabbit Cells Susceptible to Rubella Virus. *Lancet*, ii, 640.

Black, P. H., Rowe, W. P., Turner, H. C. & Huebner, R. J. (1963) A Specific Complement-fixing Antigen Present in SV40 Tumour and Transformed Cells. *Proc. natn Acad. Sci., USA*, **50**, 1148.

Collard, W., Thornton, H. & Green, M. (1973) Cells Transformed by Human Herpesvirus Type 2 Transcribe Virus Specific RNA Sequences Shared by Herpesvirus Types 1 and 2. *Nature* (*New Biol.*), **243**, 264.

Darai, G. & Munk, K. (1973) Human Embryonic Lung Cells Abortively Infected with Herpes Virus Hominis Type 2 Show Some Properties of Cell Transformation. *Nature* (*New Biol.*), **241**, 268.

Diamond, L. (1967) Two Spontaneously Transformed Cell Lines Derived from the Same Hamster Embryo Culture. *Int. J. Cancer*, **2**, 143.

Duff, R. & Rapp, F. (1971*a*) Oncogenic Transformation of Hamster Cells after Exposure to Herpes Simplex Virus Type 2. *Nature* (*New Biol.*), **233**, 48.

Duff, R. & Rapp, F. (1971*b*) Properties of Hamster Embryo Fibroblasts Transformed *In Vitro* after Exposure to Ultraviolet Irradiated Herpes Simplex Virus Type 2. *J. Virol.*, **8**, 469.

Duff, R., Doller, E. & Rapp, F. (1973) Immunologic Manipulation of Metastases due to Herpes Virus Transformed Cells. *Science*, **180**, 79.

Earle, W. R. (1943) Production of Malignancy *In Vitro*. IV. The Mouse-fibroblast Cultures and Changes seen in Living Cells. *J. natn Cancer Inst.*, **4**, 165.

Eddy, B. E., Borman, G. S., Grubbs, G. E. & Young, R. D. (1962) Identification of the Oncogenic Substances in Rhesus Monkey Kidney Cell Cultures as Simian Virus 40. *Virology*, **17**, 65.

FALK, L. A., WOLFE, L. G., HOEKSTRA, J. & DEINHARDT, F. (1972) Demonstration of Herpesvirus Saimiri-associated Antigens in Peripheral Lymphocytes from Infected Marmosets During *In Vitro* Cultivation. *J. natn Cancer Inst.*, **48**, 523.

FOX, T. O. & LEVENE, A. J. (1971) Relationship between Virus-induced Cellular DNA Synthesis and Transformation by SV40. *J. Virol.*, **7**, 473.

FRENKEL, N., ROIZMAN, B., CASSAI, E. & NAHMIAS, A. J. (1972) A Herpes Simplex 2 DNA Fragment and its Transcription in Human Cervical Cancer Tissue. *Proc. natn Acad. Sci., USA*, **69**, 3784.

GARFINKLE, B. & MCAUSLAN, B. R. (1972) Transformation of Cultured Mammalian Cells by Viable Herpes Simplex Virus, Subtypes 1 and 2. *Proc. natn. Acad. Sci., USA*, **71**, 220.

GEDER, L. & SKINNER, G. R. B. (1971) Differentiation between Type 1 and Type 2 Strains of Herpes Simplex Virus by an Indirect Immunofluorescent Technique. *J. gen. Virol.*, **12**, 179.

GEY, G. O. (1941) Cytological and Cultural Observations on Transplantable Rat Sarcoma Produced by the Inoculation of Altered Normal Cells Maintained in Continuous Culture. *Cancer Res.*, **1**, **16**, 737.

GROSS, L. (1953) A Filterable Agent, Recovered from Ak Leukaemic Extracts, Causing Salivary Gland Carcinomas in C3H Mice. *Proc. Soc. exp. Biol. Med.*, **83**, 414.

HABEL, K. (1965) Specific Complement-fixing Antigens in Polyoma Tumours and Transformed Cells. *Virology*, **25**, 55.

HENLE, W., DIEHL, V., KOHN, G., ZUR-HAUSEN, H. & HENLE, G. (1967) Herpes-type Virus and Chromosome Marker in Normal Leukocytes after Growth with Irradiated Burkitt Cells. *Science*, **157**, 1064.

HINZE, H. C. & WEGNER, D. L. (1973) Oncogenicity of Rabbit Herpes Virus. *Cancer Res.*, **33**, 1434.

HUEBNER, R. J., ROWE, W. P., TURNER, H. C. & LANE, W. T. (1963) Specific Adenovirus Complement-fixing Antigens in Virus-free Hamster and Rat Tumours. *Proc. natn Acad. Sci., USA*, **50**, 379.

HUNT, R. D., MELENDEZ, L. V., KING, N. W. & GARCIA, F. G. (1972) Herpesvirus Saimiri. Malignant Lymphoma in Spider Monkeys. *J. med. Primatology*, **1**, 114.

HUNT, R. D. & MELENDEZ, L. V. (1972) Clinical, Epidemiological and Pathologic Features of Cytocidal and Oncogenic Herpesvirus in South American Monkeys. *J. natn Cancer Inst.*, **49**, 261.

JARRET, O. & MACPHERSON, J. (1968) The Basis of the Tumourogenicity of BHK 21 Cells. *Int. J. Cancer*, **3**, 654.

KUTINOVA, L., VONKA, V. & BROUCEK, J. (1973) Increased Oncogenicity and Synthesis of Herpes Virus Antigens in Hamster Cells Exposed to Herpex Simplex Type 2 Virus. *J. natn Cancer Inst.*, **50**, 759.

LATARJET, R., CARMER, R. & MONTAGNIER, L. (1967) Inactivation of Ultraviolet X-ray and γ-irradiation of the Infecting and Transforming Capacities of Polyoma Virus. *Virology*, **33**, 104.

MACPHERSON, I. & STOKER, M. (1962) Polyma Transformation of Hamster Cell Clones—an Investigation of Genetic Factors Affecting Cell Competence. *Virology*, **16**, 147.

MACPHERSON, I. (1966) Mycoplasmas in Tissue Culture. *J. Cell Sci.*, **1**, 145.

MACPHERSON, I. & RUSSELL, W. (1966) Transformation in Hamster Cells Mediated by Mycoplasmas. *Nature, Lond.*, **210**, 1343.

MCNAB, J. C. M. (1974) Transformation of Rat Embryo Cells by Temperature Sensitive Mutants of Herpes Simplex Virus. *J. gen. Virol.*, **24**, 143.

MANAKER, R. A. & GROUPE, V. (1956) Discrete Foci of Altered Chicken Embryo Cells Associated with Rous Sarcoma Virus Tissue Culture. *Virology*, **2**, 838.

MUNOZ, N. (1972) Effect of Herpes Virus Type 2 and Hormonal Unbalance on the Uterine Cervix of the Mouse. *Cancer Res.*, **33**, 1504.

NADKARNI, J. S., NADKARNI, J. J., KLEIN, G., HENLE, W., HENLE, G. & CLIFFORD, P. (1970) EB Viral Antigens in Burkitt Tumor Biopsies and Early Cultures. *Int. J. Cancer*, **6**, 10.

NAHMIAS, A. J., NAIB, Z. M., JOSEY, W. E., MURPHY, F. A. & LUCE, C. F. (1970) Sarcomas after Inoculation of Newborn Hamsters with *Herpesvirus hominis*, Type 2 Strains. *Proc. Soc. exp. Biol. Med.*, **134**, 1065.

NAHMIAS, A. J., JOSEY, W. E., NIAB, Z. M., LUCE, C. F. & DUFFEY, A. (1970) Antibodies to *Herpesvirus hominis* Types 1 and 2 in Humans. 1 Patients with Genital Herpetic Infections. 2. Women with Cervical Cancer. *Am. J. Epidem.*, **91**, 539.

NAIB, Z. M., NAHMIAS, A. J. & JOSEY, W. E. (1966) Cytology and Histopathology of Cervical Herpes Simplex Infection. *Cancer*, **19**, 1026.

PAUL, J. (1960) *Cell and Tissue Culture.* 2nd ed. Edinburgh: Livingstone.

PLUMMER, G., GOODHEART, C. R., MIYAGI, M., SKINNER, G. R. B., THOULESS, M. E. & WILDY, P. (1974) Herpes Simplex Viruses: Discrimination of Types and Correlation between Different Characteristics. *Virology*, **60**, 206.

POPE, J. H., HORNE, M. K. & SCOTT, W. (1969) Identification of the Filterable Leucoyte-transforming Factor of QIMR-WIL Cells as Herpes-like Virus. *Int. J. Cancer*, **4**, 255.

RAWLS, W. E., TOMPKINS, W. A. F., FIGUEROA, M. & MELNICK, J. L. (1968) Herpesvirus Type 2: Association with Carcinoma of the Cervix. *Science*, **161**, 1255.

REGAN, J. D. & SMITH, J. B. (1965) Triploidy in a Human Cell Line. *Science*, **149**, 1516.

ROSS, L. J. M., WATSON, D. H. & WILDY, P. (1968) Development and Localization of Virus Specific Antigens During the Multiplication of Herpes Simplex Virus in BHK 21 Cells. *J. gen. Virol.*, **2**, 115.

ROYSTON, I. & AURELIAN, I. (1970) Immunofluorescent Detection of Herpesvirus Antigen in Exfoliated Cells from Human Cervical Carcinoma. *Proc. natn Acad. Sci., USA*, **67**, 204.

SKINNER, G. R. B., THOULESS, M. E. & JORDAN, J. A. (1971) Antibodies to Type 1 and Type 2 Herpes Virus in Women with Abnormal Cervical Cytology. *J. Obstet. Gynaec. Br. Commonw.*, **78**, 1031.

SMITH, H. S., SEBER, C. D. & TODARO, G. J. (1971) Induction of Cell Division in Medium Lacking Serum Growth Factor by SV40. *Virology*, **44**, 359.

SVET-MOLDAVSKY, G. J. (1958) Sarcoma in Albino Rats Treated during the Embryonic Stage with Rous Sarcoma Virus. *Nature, Lond.*, **182**, 1452.

TAKAHASHI, M. & YAMANISHI, K. (1974) Transformation of Hamster Embryo and Human Embryo Cells by Temperature Sensitive Mutants of Herpes Simplex Virus Type 2. *Virology*, **61**, 306.

TOOZE, J. (1973) *The Molecular Biology of Tumour Viruses*. Cold Spring Harbor Monograph Series.

TRENTIN, J. J., YABE, Y. & TAYLOR, G. (1962) The Quest for Human Cancer Viruses. *Science*, **137**, 835.

TSUDA, T. (1965) Establishment of Two Cell Lines from Syrian Hamster Embryonic Tissue *In Vitro*. *Tohuku J. exp. Med.*, **86**, 380.

VANTSIS, J. F. & WILDY, P. (1962) Interaction of Herpes Virus and HeLa Cells: Comparison of Cell Killing and Infective Centre Formation. *Virology*, **17**, 225.

WATSON, D. H., SHEDDEN, W. I. H., ELLIOT, A., TETSUKA, T., WILDY, P., BOURGAUZ-RAMOISY, D. & GOLD, E. (1966) Virus Specific Antigens in Mammalian Cells Infected with Herpes Simplex Virus. *Immunology*, **11**, 399.

WILDY, P. (1955) Recombination with Herpes Simplex Virus. *J. gen. Microbiol.*, **13**, 346.

WILKIE, N. M., CLEMENTS, J. B., MCNAB, J. C. M. & SUBAK-SHARPE, J. H. (1974) *The Structure and Biological Properties of Herpes Simplex Virus DNA*. Symposia on Quantitative Biology. Cold Spring Harbor. Vol. XXXIX **2**, 657.

YAMANE, I. & TSUDA, T. (1966) Malignant Transformation and Chromosome Change of Two Cell Lines from Syrian Hamster Embryonic Tissue *In Vitro*. *Tohuku J. exp. Med.*, **88**, 171.

ZUR-HAUSEN, H. & SCHULTE-HOLTHAUSEN, H. (1970) Presence of EB-Virus Nucleic Acid Homology in a "Virus-free" Line of Burkitt Tumour Cells. *Nature, Lond.*, **227**, 245.

ZUR-HAUSEN, H., SCHULTE-HOLTHAUSEN, H., KLEIN, G., HENLE, G., CLIFFORD, P. & LANTESSON, L. (1970) EB-Virus DNA in Biopsies of Burkitt Tumours and Anaplastic Carcinomas of the Nasopharynx. *Nature, Lond.*, **228**, 1056.