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Reduced tumorigenicity of rodent tumour cells and tumour explants following infection with wild type and mutant herpes simplex virus, bovine mammillitis virus and encephalomyocarditis virus

G.R.B. Skinner, M. Cowan, J. Davies, K. Brookes, M. Billstrom and A. Buchan
Vaccine Research Foundation, Department of Medical Microbiology, The Medical School, University of Birmingham, Birmingham B15 2TJ, UK

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Summary. The tumorigenicity of neoplastic hamster and mouse cell lines and tumour explants was reduced by infection with herpes simplex virus (HSV-1), a thymidine-kinaseless mutant of herpes simplex virus, namely 'MDK', encephalomyocarditis virus (EMC) and bovine mammillitis virus (BMV). There was an approximate relationship between duration of virus infection *in vitro* and reduction in incidence and/or rate of tumour development. The rate of tumour development was also reduced by 'site inoculation' of virus (HSV-1) at various time intervals following inoculation of tumorigenic BHK 21 cells indicating that virus was capable of reducing the rate of tumour development in a situation where the neoplastic cells were already transplanted into the susceptible host species. It is suggested that the therapeutic role of wild type, mutant or recombinant viruses merits further exploration towards prevention and treatment of human cancer.

Keywords: tumorigenicity, herpes simplex virus (HSV-1), MDK virus, encephalomyocarditis virus (EMC), bovine mammillitis virus (BMV)

It is possible that the pathological sequelae of virus infection may have led to premature discardment of the possible role of viruses in the prevention and therapy of human tumours. As an example, there is evidence that parvovirus will inhibit cell transformation by Simian virus 40 (Mousset & Rommelaere 1982) and can reduce the tumorigenicity of adenovirus or type 2 herpes simplex virus transformed cells (Ostrove *et al.* 1981; Cukor *et al.* 1975). Moreover there is evidence of suppression of Ehrlich ascites and sarcoma tumours in mice by minute virus of mice and bovine enterovirus 1 (Guetta *et al.* 1986; Taylor *et al.* 1971). While these results suggest a direct or toxic inhibition of oncogenesis, the mechanism may be more complicated as there is evidence that simultaneous inoculation of Adeno-associated virus and adenovirus 12 to hamsters (Kirschstein *et al.* (1968) reduces the incidence of adenovirus induced tumours and of particular interest

Correspondence: G.R.B. Skinner, Vaccine Research Foundation, Department of Medical Microbiology, The Medical School, University of Birmingham B15 2TJ, UK.

that pre-immunisation of new born hamsters with parvovirus will reduce the subsequent incidence of 7, 12-dimethyl benzanthracene induced and spontaneous tumours (Toolan *et al.* 1982; Toolan 1967). These are intriguing observations and may suggest long-term immunological surveillance or viral cytotoxicity for tumour cells or possibly an interference of tumour development in the cells of the animal by incorporation of virus genome into some or all the cells of the animal; while it is presently impossible to discriminate these hypotheses, the data do suggest new possibilities in the prevention of tumorigenesis.

This study explores three aspects of viral inhibition of cell oncogenicity:

(1) The effect of viruses on tumorigenic cell lines where the neoplastic potential has not been induced (as far as is known) by viruses.
(2) The effect of viruses on explants of tumour tissue.
(3) The effect on tumorigenic cell lines and explants of four viruses of varying pathogenicity for human subjects, namely type 1 herpes simplex virus, a pathogenic but usually non-fatal infection of human subjects, 'MDK' a thymidine kinaseless mutant type 1 herpes virus strain which may have limited pathogenicity for human subjects, encephalomyocarditis virus (EMC) which is not a recognised human pathogen but will replicate in human cell lines and bovine mammillitis virus (BMV) which is again not a recognised human pathogen and in addition will not replicate in human cell lines or explants (Skinner *et al.* 1987). There is evidence that infection by all four viruses can reduce the tumorigenicity of tumour cells and explants.

Materials and methods

Virus strains. The following virus strains were used. HFEM a 'classical' type 1 herpes simplex virus isolate 'MDK', a thymidine-kinaseless mutant type 1 virus strain (Dubbs & Kit 1964), the 'Allerton' strain of bovine mammillitis virus (BMV) which was kindly supplied by Professor P. Castrucci, Universita Di Perugia, Instituto Di Malattie Infettive, Perugia, Italy and encephalomyocarditis virus (EMC). Virus stocks were grown in baby hamster kidney cells and titrated by suspension plaque assay (Russell 1962).

Cells. BHK 21 (C13) cells, a stable highly tumorigenic cell line derived from a single clone (McPherson & Stoker 1962) were used for hamster inoculation, virus propagation and virus titrations.

Cell line NCTC (clone 2472), a tumorigenic mouse fibroblastic cell line was used for inoculation of mice; this cell line was obtained from Flow Laboratories (Rickmansworth, Herts, WD3 1PQ).

Virus infection of cells for animal inoculation. For hamster inoculation, BHK 21 cells were infected with 25 plaque forming units (pfu) per cell and the virus absorbed by gentle shaking for 45 min at 37°C. The cells were then washed by centrifugation three times in Eagle's medium to remove unabsorbed virus and plated out for various time intervals at 37°C. Control uninfected cells were mock infected in similar fashion to the virus infected cells. Prior to inoculation the cells were washed three times in Eagle's medium and in certain cases incubated with the appropriate anti-viral or anti-cell serum at a 1:10 dilution in Eagle's medium at 37°C for 30 min to reduce virus infectivity by virus neutralisation or by inhibition of virus release from virus infected cells (Skinner *et al.* 1975/1976). Hamsters were inoculated with 2×10^6 cells when infected with HSV and with 2×10^5 cells when infected with BMV. Animals were monitored twice weekly for tumour development.

The proportion of cells 'successfully' infected with virus was estimated by indirect immunofluorescence. Herpes virus infected cells were examined by rabbit hyperimmune antiserum against HSV-1 infected cell

extract and EMC infected cells by hyperimmune antiserum against EMC infected cell extract. Hyperimmune sera were prepared as described by Geder and Skinner (1971). Virus replication was estimated by removing samples of cells at various time intervals and titrating virus infectivity following disruption of cells by ultrasonic vibration.

Virus infection of tumour explants. Tumours were induced in hamsters and C3H mice by inoculation of 2×10^6 BHK 21 cells or NCTC 2472 cells, respectively. The tumours were removed and dissected into explants measuring approximately 25 mm^3 and infected by suspending each explant in a virus suspension in supplemented Eagle's medium at virus concentration of 5×10^6 pfu/explant for various times of incubation. Prior to animal implantation the explants were washed three times to remove unabsorbed virus and incubated with rabbit anti viral serum at 1:10 dilution for 30 min at 37°C; explants that were infected with BMV were washed without anti-serum treatment prior to implantation.

Virus replication in tumour explants was monitored by partial ultrasonic disruption of explants in 0.5 ml of sterile water followed by virus titration as described.

Animal inoculation. Tumour cells were injected subcutaneously into the dorsal aspect of the mouse or hamster. Tumour explants were injected subcutaneously through a 0.5 cm incision in anaesthetised animals. The incisions were repaired with one or occasionally two stitches.

Results

Tumour cell lines

The proportion of virus infected cells following *in vitro* infection was examined by immunofluorescence at various time intervals following infection (Table 1). In spite of the high multiplicity of the virus infection (25 plaque forming units per cell), the proportion of virus antigen positive cells was often less than 100%; indeed with BMV the proportion of infected cells was surprisingly low and had declined by the fourth day of in-vitro virus infection. This surprising result has been confirmed in several experiments and independently reported (Davies *et al.* in preparation).

Table 1. Virus antigen positive BHK-21 cells following virus infection for varying time intervals

Viruses	Duration of in-vitro virus infection (h)	Proportion (%) of virus-antigen positive cells
HSV-1	24	97·6
	48	91·2
	72	98·1
	96	99·8
MDK	24	99·8
BMV	24	33·4
	48	64·5
	72	86·8
	96	36·1
EMC	24	99·8

The proportion of virus–antigen positive cells was estimated by scanning at least fifty cells under a Dialux fluorescent microscope.

The effect of HSV-1 and BMV virus infection for various time intervals on the tumorigenicity of BHK-21 cells is shown in Tables 2 and 3, respectively. There were no tumours from HSV-1 or from BMV infected cells which had been infected for at least 24 h. In the two hamsters who received cells infected with HSV-1 for only 2 h prior to inoculation the time interval to tumour development was longer than in hamsters receiving uninfected cells (Table 2).

To investigate two other viruses—namely MDK and EMC—and the role of cell to cell spread of virus within the inoculated and progeny tumour cells, the cells were treated with $\frac{1}{10}$ dilution of hyperimmune HSV-1 or

Table 2. Tumorigenicity of BHK-21 cells infected with type 1 HSV for various time intervals

Duration of in-vitro virus infection or in-vitro suspension of uninfected cells	Uninfected		Infected	
	Proportion developing tumours	Time interval to tumour development (days)	Proportion developing tumours	Time interval to tumour development (days)
2 h	2/2	11,9	2/2	23.42
24 h	2/2	12,10	0/2	>140
48 h	—	—	0/2	>140
72 h	—	—	0/2	>140
96 h	2/2	7,7	0/2	>140
Total/means	6/6	9	2/10	>140

Table 3. Tumorigenicity of BHK-21 cells following infection with BMV for various time intervals

Duration of in-vitro virus infection or in-vitro suspension of infected cells	Uninfected		Infected	
	Proportion developing tumours	Time interval to tumour development (days)	Proportion developing tumours	Time interval to tumour development (days)
2 h	2/2	22,46	0/2	
24 h	—	—	0/2	
48 h	2/2	36,36	0/2	>140 >140
72 h	—	—	0/2	
96 h	2/2	26,26	0/2	
Total/means	6/6	32.0	0/10	>140

EMC antiserum or BHK-21 antiserum which will inhibit virus release without virus neutralization (Skinner *et al.* 1975/1976); cells were incubated with antisera for 30 min at 37°C following which the antisera were removed by three successive washes using growth medium. This dilution of anti-cell serum was shown to be not cytotoxic for BHK-21 cells in previous tests.

None of the hamsters who received virus infected cells developed tumours excepting two hamsters who had received HSV-1 infected cells pre-treated with both HSV-1 and cell antiserum and one hamster who received MDK infected cells pre-treated with HSV-1 antiserum developed tumours (Table 4); these tumours developed at 47, 52 and 66 days, respectively. All 14 hamsters inoculated with uninfected cells developed tumours within an average period of 16 days.

These experiments were repeated in mice using the tumorigenic mouse cell line NCTC 2472. Groups of three mice received cells infected with HSV, BMV or EMC for 2, 48 or 96 h. None of the mice inoculated with these

Table 4. Tumorigenicity of BHK-21 cells following infection by HSV-1, MDK or EMC; pre-inoculation treatment of cells with viral and/or cell antiserum

Virus infections	Pre-treatment of cells	Proportions developing tumours	Mean time interval to tumour development (days)
HSV1	No serum	0/3	>140
	HSV-1 antiserum	0/3	>140
	Cell antiserum	0/3	>140
	HSV-1 and cell antiserum	2/3	>140 (47,52)
MDK	No serum	0/3	>140
	HSV-1 antiserum	1/3	>140 >140 (66)
EMC	No serum	0/3*	
	EMC antiserum	0/3	>140
Total virus infected	No serum	0/6	>140
	Virus antiserum	1/9	>140 (66)
	Virus cell antiserum	2/3	>140 (47,52)
	Cell antiserum	0/3	>140
	Total	0/21 (14%)	>140
Uninfected cells	No serum	11/11	13±0.9
	Cell antiserum	3/3	30±7.9
	Total	14/14 (100%)	16±2.4

The numbers in brackets indicate the time interval to tumour development in animals who had developed tumours by 140 days.

*These three hamsters died of disseminated EMC infection within 5 days and are therefore not isolated in the column totals.

virus infected cells developed tumours by 140 days, while all 10 mice who received uninfected cells developed tumours within 10 to 24 days.

In summary, the pooled data from hamster and mice experiments (Tables 2–4) indicate that three of 44 animals (6–8%) developed tumours when inoculated with virus infected cells compared to 100% of control animals who received uninfected cells. While the time interval to tumour development by uninfected control cells was quite variable between experiments performed at different times pre-treatment of uninfected control cells with cell antiserum did to a moderate extent decrease their oncogenicity in terms of the time interval to tumour development ($P<0.05$).

The possibility that oncogenic cells might be susceptible to virus infection following their inoculation into the appropriate test animal was explored. A total of 10^6 BHK-21 cells were inoculated subcutaneously into hamsters as described and 10^7 pfu of HSV-1 or BMV inoculated into the same site at varying time intervals (Table 5). The rate of tumorigenesis was reduced by virus inoculation with an approximate relationship between cell–virus inoculation interval and rate of tumour formation. By 14 days there was a significant reduction in tumour development in the virus inoculated hamster

Table 5. Tumorigenicity of BHK-21 cells following 'site addition' of virus at varying time intervals following inoculation of cells into hamsters

Time of virus inoculation	Proportion developing tumours by 14 days		
	HSV-1	BMV	Total (HSV-1 + BMV)
Zero and 7 days	0/2	1/2	1/4
24 h and 7 days	1/2	1/2	1/4
48 h and 7 days	2/2	1/2	3/4
72 h and 7 days	1/2	2/2	3/4
96 h and 7 days	1/2	2/2	3/4
Total	5/10	7/10	12/20

Uninfected control BHK-21 cells induced 100% of tumours in 12 hamsters within 14 days.

Table 6. Replication of viruses in tumour explants

Virus	Duration of in-vitro virus infection (h)	Virus yield (pfu) per explant
HSV-1	0	6.4×10^2
	48	2.0×10^6
	72	1.6×10^6
	96	1.4×10^5
MDK	0	4.3×10^2
	24	9.3×10^5
	72	8.5×10^5
BMV	0	1.2×10^2
	24	6.2×10^2
	72	9.0×10^3
EMC	0	2.8×10^3
	24	1.3×10^6
	72	4.3×10^5

although all hamsters had developed tumours by 21 days.

Tumour explants. The replication of viruses in tumour explants *in vitro* is shown in Table 6. There was evidence of virus replication with an increase in virus titre of over 1000-fold for HSV-1 and MDK mutant strain and approximately 100-fold for EMC and BMV.

The tumorigenicity of hamster tumour explants following infection with HSV-1, 'MDK' and BMV for 1 to 4 days in organ culture prior to hamster implantation is shown in Figs 1 and 2. There was a significant delay in the rate of tumour development as measured by tumour diameter (Fig. 1) or by time interval for the tumour to reach a diameter of 7.5 mm (Fig. 2); the reduction in rate of tumour development approximately correlated with duration of in-vitro virus infection prior to explant implantation. While uninfected control tumour explants were not unaffected by the duration of in-vitro culture prior to implantation their level of tumorigenicity remained significantly higher than virus infected explants (Fig. 2). Only two hamsters had developed tumours by 14 days after inoculation of virus infected cells compared to 14 of 15 animals who received uninfected tumour explants; by 120 days, two hamsters who had received tumour explants infected with HSV-1 and BMV had not developed tumours with gradual disappearance of the implanted tumour explants.

The tumorigenicity of mice tumour explants following infection with HSV-1 is shown in Table 7. There was a delay in the rate of tumour development from virus infected cells; in one mouse, who was inoculated with an explant which had been infected for only two hours, the tumour did not develop but gradually disappeared.

Nature of tumours. Both BHK-21 and NCTC 2472 cells formed poorly differentiated sarcomata in hamsters and mice respectively. These tumours were removed and used as 'tumour explants' for implantation; there was no histological change in tumour explants following their re-implantation. There was no evidence of a significant granulomatous or lipomatous reaction to cell or explant inoculation.

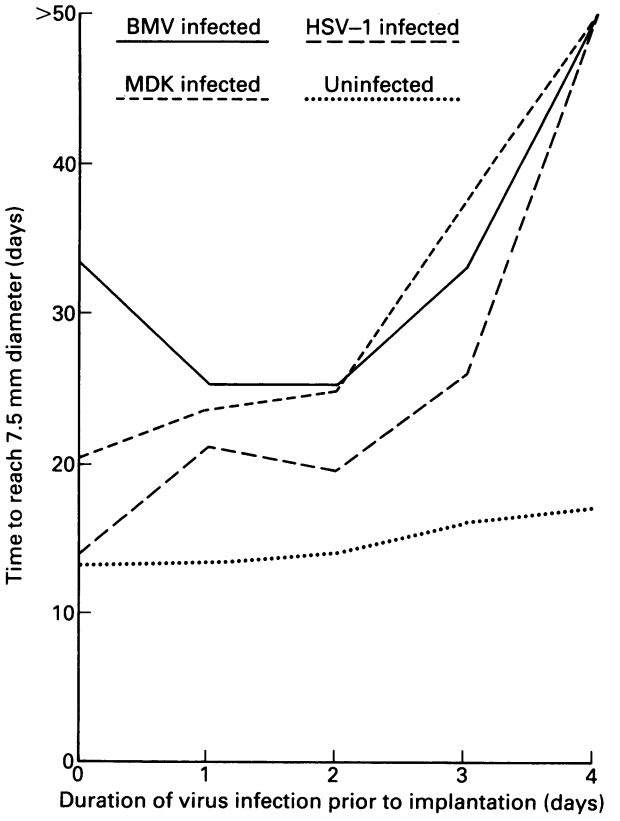

Fig. 1. Tumour size in relation to duration of in-vitro virus infection of explants.

Discussion

This study has indicated that the tumorigenicity of neoplastic cell lines and tumour explants in two animal systems can be reduced by infection with four viruses selected to represent a spectrum of pathogenicity for human subjects. In two hamsters and in one mouse, the oncogenicity of whole tumour explants was eliminated by virus infection indicating that the principle of elimination of every malignant cell in a solid tumour may indeed be viable. It appeared that continuance of virus infection of tumour cells *in vitro* may play a role in inhibition of tumour development (Table 4) as there was a suggestion that antibody treated virus infected cells may be less tumorigenic than uninfected control cells; however there was insufficient data to discriminate between an antiviral or anticellular effect particularly as anticellular sera will also inhibit virus release (Skinner *et al.* 1975/76).

There was some variation in the time interval to tumour development by uninfected control hamster cells (Tables 2–4). This variation was to be expected as a result of experimental data presented in Table 3 where there were unusually long tumour inoculation intervals as the number of inoculated BMV infected and control uninfected cells were reduced by tenfold in this experiment as it was found that 10^6 cells infected with 25 pfu per cell under these experimental conditions resulted in a rather agglutinated cell monolayer with irregular clump-

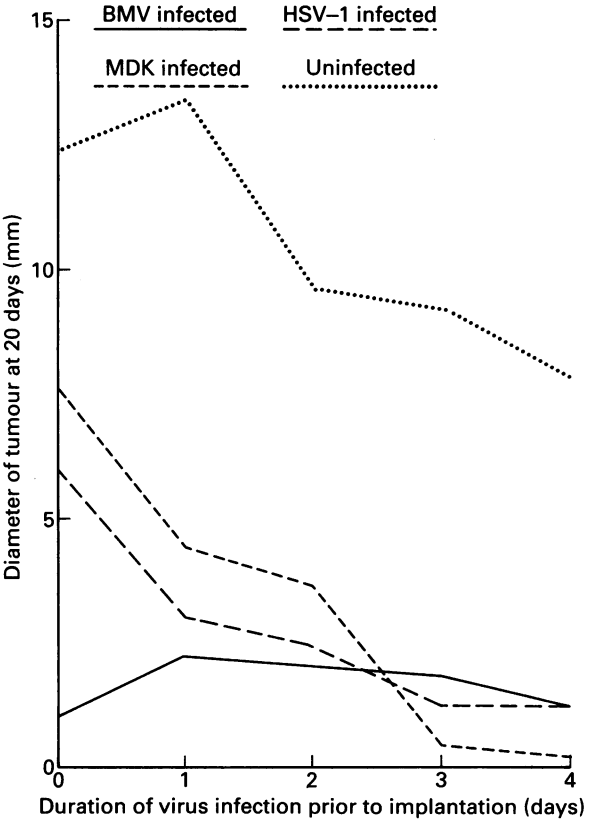

Fig. 2. Rate of tumour growth in relation to duration of in-vitro virus infection of explants.

Table 7. Tumorigenicity of mice tumour explants following infection with HSV-1

	Uninfected		Infected	
	Proportion developing tumours	Time interval to tumour development (days)	Proportion developing tumours	Time interval to tumour development (days)
2 h	2/2	18,18	1/2	>40,18
48 h	2/2	22,10	2/2	22,24
96 h	2/2	14,10	2/2	28,28
Total/means	6/6	15	5/6	>140

ing of cells which might bias the results in favour of reduced oncogenicity.

The tumorigenicity of BHK-21 cells was reduced when virus was added following inoculation of these cells into the appropriate susceptible test animal; while test control differences were small in this latter experiment it is likely that this might have been a consequence of the experimental construction where virus was inoculated somewhat 'blindly' into the erstwhile site of cell inoculation and it is possible that once a palpable, although small, tumour had developed, virus inoculation could be better 'targetted'. However, the results do indicate that neoplastic cells remain susceptible to virus infection *in vivo*. It is intended to explore the susceptibility of solid or other tumours to *in vivo* virus infection.

Inhibition of tumorigenesis might be a consequence of immune rather than virus induced cytolysis of tumour cells. This seems unlikely for the following reasons:

(1) There were test–control differences by 6–7 days by which time it is unlikely that the host animals would have mounted a significant immune response to virus antigens to which there was no previous exposure.
(2) Pre-treatment of virus infected cells with viral or cell antiserum did not further reduced tumorigenicity but marginally increased the tumorigenicity of the cells which discourages implication of immune cytolysis as a possible mechanism.
(3) In a separate study pre 'vaccination' of hamsters with inactivated BHK-21 cells or HSV infected BHK-21 cells did not inhibit the frequency of tumour development by BHK-21 cells.
(4) There is evidence that part of the mechanism is due to virus cytolysis of tumour cells as typical viral cytopathic effects have been identified in explants of hamster and mouse tumours removed 3 days following their implantation into host animals (Davies *et al.* 1988).

It appears therefore that inhibition of tumour development is mainly a consequence of virus induced tumour cell cytology. This is consistent with the general view of the mechanism of parvovirus induced inhibition of tumour development (Sprecher-Goldberger *et al.* 1970; Mayor *et al.* 1976; Georg-Fries *et al.* 1984) and with most interesting studies where pre-inoculation of newborn hamsters reduced the subsequent frequency of chemically induced or spontaneous tumours (Kirschstein *et al.* 1968; Toolan & Ledinko 1968; Toolan *et al.* 1982) and it seems unlikely that parvovirus would induce immunological protection against chemically induced or spontaneous tumours. The precise mechanism of parvovirus induced in tumour inhibition requires further investigation and resolution.

It is difficult to define strategy for human subjects; there is a general dilemma in that the less pathogenic a virus, the less cytolytic and oncolytic it will tend to be for human tumours. This is not an invariable rule and, as an example, BMV would seem to be a useful candidate for tumour inoculation in hamsters where the virus is cytolytic, inhibits the growth of hamsters cells *in vitro* prior to their inoculation, but induces only a localized infection with no evidence of neurological complications in hamster species (Skinner *et al.* 1987). It is also possible that certain pathogenic effects may be tolerable in consideration of the seriousness of the disease under treatment and this philosophy already applies with various chemotherapeutic or radiotherapeutic regimens in current use.

It is intended to further explore the role of wild type, mutant or recombinant viruses and other micro-organisms towards the prevention and/or modification of cancer in human subjects.

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References

CUKOR G., BLACKLOW N.R., KIBRICK S. & SWAN I.C. (1975) Effect of Adeno-associated virus on cancer expression by herpes virus-transformed hamster cells. *J Natl. Cancer Inst.* **55**, 947.

DAVIES J., BROOKES K., COWAN M., MOULD J., O'BRIEN M., NICHOLSON H.O., MORRISON J.M., BASU M.K., BUCHAN A. & SKINNER G.R.B. (1988) Replication of herpes viruses in explants of human and hamster tumours. Submitted to *Int. J. Can.*

DUBBS D.R. & KIT S. (1964) Mutant strains of herpes simplex deficient in thymidine kinase inducing ability. *Virology* **22**, 493.

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.

GEORG-FRIES B., BIEDERLACK S., WOLF J. & ZUR HAUSEN H. (1984) Analysis of proteins, helper dependence and seroepidermiology of a new human parvovirus. *Virology* **134**, 64–71.

GUETTA E., GRAZIANI Y. & TAL J. (1986) Suppression of Ehrlich ascites tumours in mice by minute virus of mice. *J. Natl. Cancer Inst.* **76**, 1177.

KIRSCHSTEIN R.L., SMITH K.O. & PETERS E.A. (1968) Inhibition of Adenovirus 12 oncogenicity by Adeno-associated virus. *Proc. Soc. Exp. Biol. Med.* **128**, 670–673.

MCPHERSON I. & STOCKER M. (1962) Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology* **16**, 147.

MAYOR H.D., DRAKE S., STAHMANN J. & MUMFORD D.M. (1976) Antibodies to adeno-associated satellite virus and herpes simplex in sera from cancer patients and normal adults. *Am. J. Obst. Gynecol.* **126**, 100–104.

MOUSSET S. & ROMMELAERE J. (1982) Minute virus of mice inhibits cell transformation by Simian virus 40. *Nature* **300**, 537.

OSTROVE J.M., DUCKWORTH D.H. & BERNS K.I. (1981) Inhibition of Adeno virus transformed cell oncogenicity. *Virology* **113**, 521.

RUSSELL W.C. (1962) A sensitive and precise plaque assay for herpes virus. *Nature* **195**, 1028.

SKINNER G.R.B., MUSHI E.Z. & WHITNEY J.E. (1975/1976) Immune inhibition of virus release from herpes simplex virus-infected cells. *Intervirology* **6**, 296.

SKINNER G.R.B., BUCHAN A., DURHAM J., COWAN M., DAVIES J., BROOKES K. & CASTRUCCI G. (1987) The role of bovine mammillitis virus towards preparation of an alternative vaccine against herpes simplex virus infections of human subjects. *Vaccine* **5**, 55.

SPRECHER-GOLDERBERGER S., DEKEGEL D., OTTEN J. & TUIRY L. (1970) Incidence of antibodies to adeno-associated viruses in patients with tumours and other diseases. *Arch Ges. Virusforsch.* 1970 **30**, 16–21.

TAYLOR M.W., CORDELL B., SOUTHRADA M. & PRATHER S. (1971) Viruses as an aid to cancer therapy: regression of solid and ascites tumours in rodents after treatment with bovine enterovirus. *Proc. Natl. Acad. Sci.* **68**, 836.

TOOLAN H.W. (1967) Lack of oncogenic effect of the H-viruses. *Nature* **214**, 1036.

TOOLAN H.W. & LEDINKO N. (1968) Inhibition by H-1 virus the incidence of tumours produced by adenovirus-12 in hamsters. *Virology* **35**, 475–478.

TOOLAN H.W., RHODE S.L. & GIERTHY J.F. (1982) Inhibition of 7, 12-Dimethylbenz(a)anthracene-induced tumours in Syrian hamsters by prior infection with H-1 parvovirus. *Cancer Res.* **42**, 2552.