Chronic Production of Defective-interfering Particles by Hamster Embryo Cultures of Herpesvirus Persistently Infected and Oncogenically Transformed Cells

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SUMMARY

Highly passaged defective-interfering (DI) particle preparations of equine herpesvirus type 1 (EHV-1) were found to mediate the co-establishment of persistent infection and oncogenic transformation of permissive hamster embryo cells. Four cell lines, designated DI-1 to DI-4, were shown to possess biological properties typical of transformed cells and to induce the rapid formation of metastatic fibrosarcomas when injected into syngeneic LSH hamsters. Corresponding DI tumour cell lines, designated DI-1T to DI-4T, were found to be virus non-producing, to be transplantable in the hamster, and, like the four parent DI cell lines, to express EHV-1-coded antigens and to be resistant to superinfection with EHV-1 but not with a heterologous virus, vesicular stomatitis virus. All transformed cell lines, but not the tumour cell lines, contained a population of cells (2.6 to 9%) that continued to release infectious virus after 100 passages in culture. The production of interferon and selection of temperature-sensitive mutants did not appear to play a role in the maintenance of persistent infection. However, it was demonstrated that these persistently infected cells continued to release not only infectious virus but also DI particles after more than 2 years in culture. DI particles were shown to be present in released virus by: (i) detection of the defective virus DNA species (density 1.724 g/ml; standard EHV-1, density 1.716 g/ml) by CsCl analytical ultracentrifugation techniques; (ii) measurement of interference activity of virus released from DI-1 to DI-4 cells against standard EHV-1 replication; (iii) the presence of the 35 megadalton BgIII fragment unique to the DI particle genome in DNA of released virus. These studies indicate that herpesvirus DI particles may function both in the initiation and maintenance of persistent infection and alter the cytocidal effects of infection to allow the establishment of oncogenic transformation and persistent infection.

INTRODUCTION

Infection of animal cells with herpesviruses can result in either a productive infection, a non-productive infection in which the cell survives but retains the virus genome (Roizman, 1972; Spear & Roizman, 1980; Stevens, 1980) or, under certain conditions, establishment of persistent infection (Walker, 1964; Stevens & Cook, 1974; Mocarski & Stinski, 1979;
In addition, some herpesviruses have been shown to have oncogenic potential and can transform cells *in vitro* (Robinson *et al.*, 1980a, b; Rapp, 1980). The earliest findings of the simultaneous occurrence of oncogenic transformation and persistent infection were reported for papovaviruses (Dulbecco & Vogt, 1960; Koprowski *et al.*, 1962; Shein & Enders, 1962; Girardi *et al.*, 1965). More recently, herpesviruses such as human cytomegalovirus (Geder *et al.*, 1976), Epstein–Barr virus (EBV; Epstein & Achong, 1977), equine herpesvirus type 1 (EHV-1; Robinson *et al.*, 1980b) and equine cytomegalovirus (ECMV; J. Staczek, J. H. Wharton, S. A. Dauenhauer and D. J. O’Callaghan, unpublished results) have been shown to mediate persistent infection and oncogenic transformation.

In permissive cells infected with animal viruses, three mediators have been reported to be involved in the establishment and maintenance of persistent infection. The isolation of temperature-sensitive (ts) mutants from persistently infected cells, and also the establishment of persistence by ts virus preparations, have implicated their possible role in cells infected with Newcastle disease virus (NDV; Preble & Youngner, 1973a, b, 1975), reovirus (Ahmed *et al.*, 1980), vesicular stomatitis virus (VSV; Wagner, 1974; Stanners & Goldberg, 1975) and other viruses (Dal Canto & Rabinowitz, 1981). In long-term persistence in BHK21 cells established using VSV virions and defective-interfering (DI) particles, Holland *et al.* (1976) found that ts and small plaque mutants evolved which contributed to the stability of persistence. For many years a role for interferon in establishment and/or maintenance of persistent infections has also been proposed by several investigators working with influenza virus type A, NDV, mumps virus (Henle *et al.*, 1959), poliovirus (Ho & Enders, 1959), vaccinia virus (Glasgow & Habel, 1962), Sindbis virus (Enzmann, 1973) and VSV (Nishiyama, 1977). Recently, Sekellick & Marcus (1978, 1979) have proposed that induction of interferon may occur by DI particles or by ts mutants, and that these dual factors may play a synchronous role in VSV persistence. The involvement of DI particles as a mediator of persistent infection has been demonstrated in model systems using rhabdoviruses (Reichmann & Schnitzlein, 1979; Andzhaparidze *et al.*, 1981), influenza viruses (De & Nayak, 1980) and togaviruses (Stollar, 1979). Recently Roux & Holland (1979) showed that persistently infected BHK21 cells established with preparations of DI particle-rich Sendai virus contained defective RNA in amounts that were 100-fold greater than standard virus RNAs.

Earlier studies in our laboratory have demonstrated the oncogenic transformation potential of u.v.-irradiated standard EHV-1 in cultures of primary hamster embryo (HE) cells (Robinson *et al.*, 1980a). In later work, infection of HE cells with non-irradiated, purified virus enriched for low-passage EHV-1 DI particles was shown to result in the simultaneous occurrence of oncogenic transformation and persistent infection (Robinson *et al.*, 1980b; O’Callaghan *et al.*, 1981). Careful examination of the virus produced by these persistently infected hamster cells revealed the production of standard EHV-1 exclusively.

In this paper, we show that highly passaged EHV-1 defective particles, whose genome is comprised of reiterated sequences originating from the S region, possess the capacity to establish persistent infection and oncogenically transform permissive hamster embryo cells, and demonstrate that DI particles are chronically released from these persistently infected cells. Thus, this is the first report that defective particles may function in both the establishment and maintenance of herpesvirus persistent infection.

**METHODS**

**Cells.** The L-M strain of mouse fibroblasts was grown in suspension cultures in YeLP medium (yeast extract, lactalbumin hydrolysate, peptone–Eagle’s minimal essential medium) supplemented with 3% foetal bovine serum (FBS; Flow Laboratories) as described previously (O’Callaghan *et al.*, 1968a, b). Cultures of primary HE cells were prepared from the LSH
inbred hamster strain (Charles River, Lakeview, Wilmington, Mass., U.S.A.) and were grown in Eagle's minimal essential medium (MEM; Microbiological Associates, Walkersville, Md., U.S.A.) as described previously (Robinson et al., 1980a). All DI-transformed cells and tumour-derived cell lines were grown in MEM supplemented with 3% FBS. Tumour cell lines were established from sarcoma tissues as described previously (Robinson et al., 1980a).

**Viruses.** Standard EHV-1 (Kentucky A strain) was grown in L-M cell suspension cultures as described earlier (Perdue et al., 1974; Henry et al., 1979). Standard virus was passaged at low m.o.i. (0-001) and was shown to contain only standard EHV-1 DNA (density 1.716 g/ml) by CsCl isopycnic analysis in the Beckman model E analytical ultracentrifuge (Henry et al., 1979, 1980). The Indiana strain of VSV used in superinfection and interferon experiments was a gift from Dr M. Hunt. The virus was grown in L-M cells and titred by plaque assay.

**Generation of EHV-1 DI particles and virus purification.** The methods for the generation of EHV-1 DI particles have been described previously (Henry et al., 1979, 1980; O'Callaghan et al., 1981). L-M cells were infected with standard EHV-1 at an m.o.i. of 20. Following a 2 h attachment period, the cells were collected by low-speed centrifugation and resuspended in YeLP medium at a concentration of 2 \times 10^6 cells/ml. The cell-free supernatant from this infection (designated D-0) was collected at 48 h post-infection and was used undiluted as the source of virus for the next passage (designated D-1). In this and in all subsequent serial passages, 1 \times 10^9 cells were infected with 200 ml supernatant from the previous high multiplicity passage. Infections were allowed to proceed until cytopathology was judged to be maximal (48 to 72 h). The remaining supernatant at each passage was used as a source for purified virions. Purification was by polyethylene glycol 6000 precipitation of the cell-free supernatant virions followed by two or more cycles of rate-velocity centrifugation in dextran-10 gradients (Perdue et al., 1974).

**Infection of HE cells.** A purified virus preparation obtained from high multiplicity, serial passage D-16 (68% defective as evaluated by measurement of the amount of DI particle DNA (density 1.724 g/ml) in the analytical ultracentrifuge] was used to infect cultures of primary HE cells. Virus preparations were u.v.-irradiated as described by Robinson et al. (1980a) to reduce but not obliterate infectivity. Confluent cell monolayers grown in 25 cm² plastic culture flasks were each infected with 3 \times 10^9 purified virus particles resuspended in 0.1 ml distilled water. Virus was allowed to adsorb for 2 h at 37 °C after which time the cells were fed with MEM supplemented with 10% FBS.

**Cell transformation assays.** At various passages the DI-transformed cell lines were assayed for properties of biological transformation as described by O'Callaghan et al. (1981).

**Assays for virus production and examination of the virus DNA.** Culture supernatants and cell lysates of DI-transformed and tumour cells were examined for the release of EHV-1 virus particles by both plaque assay on L-M cells and by electron microscopy as described by Perdue et al. (1975). Infectious centre assays were performed to ascertain the percentage of virus-producing cells as described by Robinson et al. (1980a).

Examination of the DNA species present in virus particles released from DI-transformed cells was performed by CsCl density-gradient analysis (Campbell et al., 1976; Henry et al., 1979). Supernatant culture fluids from each DI-transformed cell line were separately pooled, clarified at 5000 rev/min for 20 min, and the virus was pelleted at 31000 g for 1 h. Virus pellets were resuspended in TE buffer (0.01 M-tris and 0.001 M-EDTA pH 7.4). The phenol-extracted virus DNA or intact particles lysed with Sarkosyl in the ultracentrifuge cell were mixed with CsCl to a final density of 1.700 g/ml and centrifuged in an AnD rotor in the Beckman model E analytical ultracentrifuge at 140000 g for 24 h at 22 °C. The gradients were photographed and buoyant densities of virus DNAs were determined by previously reported procedures (Soehner et al., 1965; Henry et al., 1979).
Restriction enzyme analysis of DNA of released virus. Virus particles prepared as described above were resuspended in TE buffer and were treated with SDS (final concn. 1%) and Pronase (1 mg/ml) for 4 h at 37 °C (Huang, 1973; Cohen et al., 1975; Henry et al., 1979). Following incubation, the DNA was extracted three times with 80% phenol in TE buffer and, in the final extraction, with equal volumes of phenol and chloroform–isoamyl alcohol (24:1, v/v). The DNA was then precipitated overnight at −20 °C in 3 vol. 95% ethanol and was resuspended in TE buffer. Aliquots of each of the DNAs were digested overnight with the restriction endonuclease BglII (3 units/μg), and the fragments were electrophoresed in 0-65% agarose (SeaKem, Marine Colloids, Rockland, Me., U.S.A.) slab gels. The separated fragments were visualized by staining with ethidium bromide and viewing under u.v. illumination (Wharton et al., 1981).

Interference assay of virus released from persistently infected cells. Suspensions of 1 × 10⁸ L-M cells were either mock-infected or were infected with 2 ml supernatant medium from persistently infected HE cells in a total 5 ml vol. Following attachment for 1.5 h at 37 °C, all cells were collected and infected with 2 p.f.u./cell of standard EHV-1 in a 5 ml vol. After incubation the cells were washed (four times with 20 ml changes of YeLP medium), resuspended (2 × 10⁶ cells/ml) in YeLP medium and incubated at 37 °C. A 1 ml sample of each cell suspension was collected immediately and then at 24-h intervals for 72 h; samples were frozen at −70 °C and assayed later for virus by the plaque method.

Assay for interferon activity. Supernatants from normal HE cells or EHV-1 persistently infected cultures were heated to 56 °C for 30 min. Confluent 25 cm² HE cultures, washed once with phosphate-buffered saline (PBS), were incubated with 5 ml of one of the heat-treated supernatants for 24 h at 37 °C. The cells were washed twice with PBS and were challenged with 100 p.f.u. of VSV. Following attachment for 2 h at 37 °C, the cell cultures were washed (four times with PBS), fed with 5 ml MEM and were sampled (1 ml) at 2 h post-infection and at 24-h intervals for 72 h. Samples were frozen at −70 °C and assayed for infectious virus by plaque titration. Positive controls were performed and employed interferon present in the serum of LSH hamsters at 22 h post-infection with 1 × 10⁴ p.f.u. of VSV.

Virus induction by treatment with 5-iodo-2'-deoxyuridine (IdUrd). Cultures of DI-transformed and DI-tumour cells were incubated for 24 h at 37 °C in MEM with 3% FBS supplemented with 25 μg/ml IdUrd. Both IdUrd-treated cells and control cells were washed three times with PBS and fed with MEM. Cell supernatants were sampled daily and virus induction was monitored by plaque assay.

Tumour pathology. Biopsy specimens of tumour tissue and major organs were taken for (i) histopathological examination by light microscopy after fixation, sectioning and staining by standard histological methods, (ii) electron microscopic studies to identify tumour cell type and monitor for subvirus and virus particles (O'Callaghan & Randall, 1976; O'Callaghan et al., 1978), and (iii) establishment of tumour cell lines by methods described above for establishing embryo cell cultures.

Indirect immunofluorescence. The preparation of antiserum to EHV-1 proteins using extracts of standard EHV-1-infected HE cells as antigen was described earlier (Robinson et al., 1980a). Semi-confluent monolayers of DI-transformed and DI-tumour cells grown on 18 × 75 mm coverslips were washed once with PBS and then fixed with methanol for 10 min at −20 °C. Cells were washed three times with PBS and exposed to hamster anti-EHV-1 antiserum (diluted 1:8 in PBS) for 1 h at 37 °C in a humidified atmosphere. After washing three times with PBS the cells were stained with fluorescein-conjugated rabbit anti-hamster IgG (1:8 dilution in PBS; Gibco), incubated for 1 h, washed as before, mounted on glass slides in a PBS–glycerol (1:1) mixture and examined with a Leitz Ortholux II fluorescent microscope equipped with Xenon-transmitted u.v. illumination.
Herpesvirus persistent infection

Fig. 1. Comparison between infectious virus titres of serial, high-multiplicity (DI) passages and interference capacity of the serially passaged virus (passages D-7 to D-14) with standard EHV-1 replication. (a) DI particles of EHV-1 were generated by serial, high multiplicity passage in L-M cells as described in Methods. (b) Interference capacity of serially passaged virus. To measure interference capacity, L-M cells were either mock-infected or resuspended in 10 ml cell-free supernatant from the DI passage and incubated for 1.5 h. Cells were collected and challenged with standard EHV-1 (m.o.i. 1 to 3). Following attachment for 1.5 h, the cells were collected and resuspended in growth medium at 2 x 10^6 cells/ml. Percentage interference with standard EHV-1 by the serially passaged virus represents the infectious titres obtained 48 h post-infection in mixed infections as compared with titres of control cells infected only with standard virus.

RESULTS

Generation of EHV-1 DI particles in L-M cells

EHV-1 DI particles were generated by serial, undiluted passage of the virus in L-M cell suspension cultures as detailed in Methods. Serial, undiluted passage of EHV-1 resulted in the cyclic appearance of infectious virus as shown by the von Magnus curve typical of serial passage of DI particles (Fig. 1a). A profile of the interference activities of the serially passaged virus in these same passages is presented in Fig. 1(b). In these experiments 1 ml of the cell-free passage supernatant was reacted with 1 x 10^8 L-M cells followed by challenge with standard EHV-1 (m.o.i. of 2). The L-M cell supernatants were sampled at 24-h intervals and the virus was assayed to monitor reductions in infectivity (i.e. interference activity) as compared to titres of controls which received only the standard virus challenge. As can be seen, standard virus replication was inhibited by particles present in passages D-7 to D-10 from 60 to 90% while particles present in passages D-11 and D-12 did not interfere. Interference activity of passages D-13 and D-14 exceeded values of 50%. Due to the cyclic production of DI particles, interference activity varied inversely with the infectious virus titre. These data and those of density analyses in the analytical ultracentrifuge of the DNA species present in the serially passaged virus [i.e. the ratio of standard EHV-1 DNA (density 1.716 g/ml) to DI DNA (density 1.724 g/ml); Henry et al., 1979] confirmed the generation of EHV-1 DI particles and identified those passages which had the greatest abundance of defective virus.

Establishment of persistently infected HE cell lines

Virus purified from serial high multiplicity passage D-16, which was found to contain 68% virus DNA having a density of 1.724 g/ml, was used to infect cultures of primary HE cells as described in Methods. These cells displayed very limited EHV-1 cytopathic effects (c.p.e.), and within 14 days post-infection four of the cultures contained several microscopic, isolated
Table 1. Properties of EHV-1 persistently infected transformed cells

<table>
<thead>
<tr>
<th>Property</th>
<th>Control HE cells</th>
<th>DI-1</th>
<th>DI-2</th>
<th>DI-3</th>
<th>DI-4</th>
</tr>
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<tbody>
<tr>
<td>Morphology</td>
<td>Fibroblastic</td>
<td>97% Fibroblastic</td>
<td>96% Fibroblastic</td>
<td>93% Fibroblastic</td>
<td>94% Fibroblastic</td>
</tr>
<tr>
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<td>&gt;100</td>
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<td>Virus production</td>
<td></td>
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<tr>
<td>p.f.u./ml††</td>
<td>0</td>
<td>1·1 x 10⁴</td>
<td>4·4 x 10⁴</td>
<td>3·3 x 10⁴</td>
<td>5·3 x 10⁴</td>
</tr>
<tr>
<td>Infectious centres‡</td>
<td>0</td>
<td>3·9</td>
<td>4·6</td>
<td>8·1</td>
<td>4·3</td>
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<tr>
<td>Electron microscopy‡</td>
<td>0</td>
<td>2·6</td>
<td>3·2</td>
<td>7·0</td>
<td>9·0</td>
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<tr>
<td>Superinfection§</td>
<td></td>
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<tr>
<td>EHV-1</td>
<td>+</td>
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<tr>
<td>VSV</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Oncogenicity‖</td>
<td></td>
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<tr>
<td>Newborn</td>
<td></td>
<td>50% Hepatitis</td>
<td>100% Sarcoma</td>
<td>100% Sarcoma</td>
<td>50% Hepatitis</td>
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<tr>
<td>Adult (+ 4 weeks)</td>
<td></td>
<td>50% Sarcoma</td>
<td>100% Sarcoma</td>
<td>100% Sarcoma</td>
<td>50% Sarcoma</td>
</tr>
<tr>
<td>Interferon production†</td>
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<td></td>
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</table>

* Number of passages which cells survived in culture.
†† Virus titres (p.f.u./ml) of passage 35 cell cultures. Titres varied from 5 x 10⁴ to 1 x 10⁵ p.f.u./ml for 75 cm² cultures; total vol. 25 ml.
‡ The percentage of total cells producing virus was measured by infectious centre assays and examination of cells in the electron microscope as described in Methods.
§ Confluent cultures were infected with 10 p.f.u./cell of either EHV-1 or VSV. Cell supernatant fluids were sampled at 2, 24 and 48 h post-infection and titred by plaque assay on L-M cells. EHV-1 titres in DI cells did not increase above the unchallenged controls (−). VSV infection produced extensive cytolysis in DI cultures within 48 h post-infection and virus replicated to significant titre (420- to 7000-fold increase) (+).
‖ Groups of 10 LSH hamsters, newborn or adult (+ 4 weeks), were inoculated subcutaneously with 5 x 10⁶ cells. Procedures for histopathological examination of tumours are described in Methods. Inoculation of animals with normal HE cells failed to induce pathological effects (−).

Areas of very dense cell growth. By 18 days post-infection these foci became easily discernible to the unaided eye and more numerous in the culture with successive cell subcultivations. These four cultures, designated DI-1 to DI-4, were chosen from eight independently established cultures for further study. By passages 7 to 10 all cultures exhibited a mixed cell morphology consisting of numerically predominant (93 to 97%) fibroblasts and a small population of rounded cells which contained the bulk of virus-coded antigens as detected by immunofluorescence (see below). All cell cultures have maintained these two cell types for more than 2 years in culture and after more than 450 cell divisions.

All four DI cell lines were found to release infectious virus, and the percentage of cells releasing virus as determined by both infectious centre assays and quantification of virus-containing cells using electron microscopy did not exceed 9% (Table 1). Further increases in virus production could not be attained by treatment of the cells with IdUrd (Shapiro et al., 1978). The nature of this virus will be presented below. All four cell lines were found to be immortal (+ 100 subcultivations) and to exhibit the biological properties of transformed cells as, in contrast to normal HE cells, these cells had reduced generation times, increased saturation densities and required less serum for normal growth. In addition, all four DI cell lines were resistant to superinfection with EHV-1 at multiplicities of 10 p.f.u./cell, and infection with the homologous virus did not alter the morphology and viability of the two cell types. In contrast, infection with VSV resulted in rapid cytolysis and production of high titres of this heterologous virus.

To ascertain whether the EHV-1 persistently infected cultures produced significant quantities of interferon, which has been shown to play a role in persistency (see Introduction), supernatants from these cultures and control HE cells were heated at 56 °C for 30 min and
Fig. 2. Oncogenicity of DI persistently infected transformed cell lines and tumour cell lines in syngeneic LSH hamsters. (a) Typical tumour-bearing hamster 6 weeks post-injection with DI-4 cells. Tumour metastasis following injection of DI cell lines and DI tumour cell lines was found in the heart (b), and a haematoxylin and eosin-stained section of the heart is shown in (c). Metastasis of tumour is shown in the lungs (d), and a haematoxylin and eosin-stained section of lung tissue with invading tumour tissue is shown in (e). Metastasis was found in the kidneys (f), and a haematoxylin and eosin-stained section of kidney shows tumour metastasis (g).
assayed for interferon activity using VSV as challenge virus. Confluent 25 cm² cultures of normal HE cells were incubated with either heat-treated growth medium (control) or supernatant from the DI cell cultures, and after 24 h the monolayers were challenged with 100 p.f.u. VSV. The finding that VSV replicated to high titres which were similar to those of control cultures indicated that supernatants of DI cell cultures did not contain antiviral activity (Table 1). These data suggest that interferon does not play a significant role in the maintenance of persistent infection in this herpesvirus system.

**Oncogenicity of DI-transformed cells**

The pathological manifestations in both newborn and immunocompetent, adult (4-week-old) LSH hamsters were examined following subcutaneous injection of each of the four persistently infected cell lines (Table 1). Although 50% of the newborn animals injected with two of the DI cell lines succumbed to a lethal hepatitis within 48 h, all remaining newborn animals and all adult animals developed solid, metastatic fibrosarcomas within 6 weeks (Table 1). In some experiments, some adult animals injected with DI-2 cells developed a soft or spongy fibrosarcoma. Fig. 2(a) shows a typical tumour-bearing animal at 6 weeks following injection with DI-4 cells. Tumour masses of this nature equivalent in size to one-third to one-half the total animal body weight were common. The examination of major organs on autopsy revealed that tumour metastasis occurred initially in the lung (Fig. 2d, e) followed by involvement of the kidneys (Fig. 2f, g) and heart (Fig. 2b, c). An accompanying splenomegaly and splenic extramedullary haematopoiesis was also observed (not shown). Surgically excised tumour tissues were used to establish the four tumour cell lines, designated DI-1T, -2T, -3T and -4T, corresponding to each parent DI-transformed cell line. All tumour cell lines were transplantable and gave rise to tumours which were histopathologically identical to those produced by the parent cell line.

All tumour cell cultures were found to be virus-negative as judged by the failure of virus plaque development when cell supernatants were incubated on L-M cell monolayers, by the absence of c.p.e. in L-M cells either co-cultivated with tumour cells or incubated with tumour cell lysates and by failure to detect virus particles in electron microscopic examinations. In addition, all four tumour-derived cell lines were found to be resistant to superinfection with standard EHV-1 but were susceptible to VSV infection; VSV titres as great as $2.5 \times 10^8$ p.f.u./ml were obtained at 48 h post-infection following an initial input of 100 p.f.u. per 25 cm² confluent cell monolayer.

**Detection of virus-coded antigens in DI-transformed and tumour-derived cells by immunofluorescence**

To demonstrate the role of EHV-1 in the transformation of these cells and to ascertain whether virus-coded proteins are produced, DI-transformed and tumour cells were examined for virus proteins by indirect immunofluorescence. Results typically obtained for DI-transformed cell lines and corresponding tumour-derived cell lines revealed that all DI-transformed and tumour cells express virus proteins. In the case of the transformed cells, both the fibroblastic and rounded cell types expressed virus proteins which were localized largely in the cytoplasmic and perinuclear regions. The small population of rounded cells which are virus producers expressed significantly greater amounts of EHV-1 antigen(s) as evidenced by the greater intensity of fluorescence. The virus non-producing tumour cell lines manifested staining characteristics similar to those of the transformed cells and provided evidence that these cells also possessed EHV-1 antigens. Quantitative estimates suggested that at least 25% of the cells in the DI-transformed cultures and at least 50% of the cells in the DI-tumour cell cultures contain EHV-1-coded protein(s).
Herpesvirus persistent infection

Fig. 3. *BglII* restriction endonuclease analysis of DNA from virus particles released from persistently infected transformed DI cell lines. Lane (a), digestion of EHV-1 standard DNA yielding 16 fragments ranging from 24.5 to 1.0 md in size; lane (b), DNA preparation from serially passaged virus with 75% DI particles containing a 35 md fragment (arrow) unique to defective DNA. Digestion of virus DNAs from particles released from DI cell lines 1 to 4 (lanes c to f respectively) also contain the 35 md fragment found in DI particle DNA.

**Nature of the virus released from DI persistently infected transformed cell lines**

Since interferon was not produced by the DI cell lines (see above), experiments were conducted to determine whether the DI persistently infected-transformed cells produced EHV-1 DI particles which may function in maintaining the state of virus persistence as
reported for other animal viruses (see Introduction). Three experimental approaches were employed to assay for the continued production of DI particles by these persistently infected cells. Initially, interference assays using supernatant fluids of DI cell lines were performed in L-M cells. Virus released from three of the DI cell lines was found to inhibit standard virus replication from 21 to 81%. Only supernatant from DI-3 cells failed to interfere with standard EHV-1 replication. Since these data provided evidence for the presence of DI particles, it was decided to examine the DNA species present in particles released by these cells by restriction endonuclease digestion, and to compare the fragment profiles obtained with the known cleavage patterns for standard EHV-1 DNA and DNA from DI preparations known to contain at least 70% defective particles. Amounts of 2 to 3 l of supernatant fluid were collected and pooled for each cell line. The virus particles were pelleted from the supernatants, and the DNA was phenol-extracted and resuspended in TE buffer following precipitation with 95% ethanol. The DNAs were digested with the restriction endonuclease BglII, and the resultant fragments were electrophoresed in 0.65% agarose slab gels. For comparison, standard EHV-1 DNA and virus DNA abundant in the defective species were similarly digested and included in adjacent lanes of the gel. The digestion products were stained with ethidium bromide and visualized by u.v. illumination. Profiles of the fragments from standard EHV-1 DNA (lane a) and DNA from a virus preparation that contained 75% DI particles (lane b) are shown in Fig. 3. As expected, standard EHV-1 DNA (lane a) yielded 16 major BglII fragments that ranged from 24.5 to 1.0 megadaltons (md) in size (Henry et al., 1981), whereas the mixture of standard and defective DNAs (lane b) contained a large 35 md fragment that comprises the bulk of the defective genome and which originates from the S region of standard DNA (O'Callaghan et al., 1981; S. A. Dauenhauer et al., unpublished results). The fragment profiles of DNA from particles released from all four DI cell lines (lanes c to f) were similar to that of the mixed DNA preparation and contained the 35 md BglII fragment unique to DI particle DNA. Thus, the finding of this large mol. wt. fragment, similar to that of DI DNA (lane b), in each of these DNAs confirms the presence of DI particles among the virions released from these persistently infected cell lines.

Lastly, CsCl isopycnic analyses of these virus DNA preparations in the analytical ultracentrifuge revealed the presence of the 1.724 g/ml species of defective DNA in all four DNA preparations. Quantification of the percentage of DI particles present in the released virus was attempted by measuring the relative amounts of the 1.724 g/ml (defective) and 1.716 g/ml (standard) virus DNAs by laser scanning of photographs obtained in these density measurements in the Beckman model E analytical ultracentrifuge. Although the amount of defective DNA present was insufficient for precise measurements, it was found that the 1.724 g/ml species was present in small amounts and comprised less than 10% of total virus DNA.

DISCUSSION

In the present investigation we have shown that EHV-1 DI particles possess significant biological activity since they are able, when present with standard virus, to initiate the co-establishment of persistent infection and oncogenic transformation of permissive hamster embryo cells. These cultures were found to exhibit properties typical of oncogenically transformed cells, and all cell lines studied were able to initiate the formation of metastatic fibrosarcomas in adult, syngeneic LSH hamsters. The simultaneous occurrence of persistent infection in these biologically transformed cells is significant in that it provides an experimental system to study the altered responses of persistence and oncogenic transformation mediated by a member of this important virus family. Although not fully understood, persistent infection of cells is commonly recognized to be mediated by one or more of three factors: the presence of interferon, an endogenous subpopulation of ts mutants and/or the presence of DI particles.
Based on these observations from other virus–cell systems, studies were performed to examine whether one or more of these factors could be identified in the four DI cell lines. Experiments designed to detect interferon in the EHV-1 persistently infected transformed cells using a VSV–HE cell assay system indicated that detectable levels were not present in the DI-1 to DI-4 cells. Preliminary findings indicate that virus produced by the persistently infected hamster cells does not differ markedly from standard EHV-1 in growth kinetics at various temperatures. To date, no evidence has been obtained for the generation of ts mutants in these persistently infected cell lines.

The continued production of DI particles in these persistently infected cells was demonstrated by three experimental approaches. (i) Virus released from DI cell lines contained DI DNA, as judged by restriction enzyme analysis. (ii) The released virus particles had interference activity against standard EHV-1 replication. (iii) Cell-released virus possessed the heavy DNA species shown to be defective DNA (Henry et al., 1979; O'Callaghan et al., 1981). Thus, this study is the first demonstration that DI particles are continuously produced in herpesvirus persistent infection and may indicate that defective virus is important, perhaps necessary, to maintain this state of persistence.

The failure to detect a subpopulation of DI particles in the cells described by Robinson et al. (1980b) could have been influenced either by one or both of two factors. Firstly, the EHV-1-transformed hamster cells described here were established by infection with DI particles that had been propagated for 16 consecutive passages whereas those established previously were transformed by low passages of DI particles from the same series (Robinson et al., 1980b). Our earlier findings (Henry et al., 1979) suggested that with continued passage EHV-1 DI particles became 'more defective', i.e. (i) the magnitude of the cyclic fluctuations in infectious virus titre increased with continued passage, (ii) the number of particles required to cause a specific reduction in virus titre decreased with continued passage, and (iii) the genomes of the DI particles became genetically less complex with continued passage. Thus, multiply-passaged DI particle preparations may contain particles whose genome has evolved to a stable genetic structure that has a selective advantage for replication, and thus, for survival and continued production in persistently infected cells. The second factor which may account for the lack of DI particle production in cells transformed with low DI particle passages is that those cultures arose from selected dense foci which grew rapidly after infection. These cells may not have contained DI particles or they may have lost the capacity to produce DI particles in the selection process, whereas the DI persistently infected transformed cells described in the present study were propagated from the total primary hamster cell culture and these conditions may have allowed the selection of cells that produce DI particles.

Lastly, our finding that the genome of the DI particles used in this study is composed only of S region sequences (O'Callaghan et al., 1981), considered in the light of our recent discovery that an L region sequence at map position 0.32 to 0.40 is associated with oncogenesis (see O'Callaghan et al., 1981; Robinson & O'Callaghan, 1981; Robinson et al., 1981), has allowed us to offer a model for the role of DI particles in herpesvirus oncogenic transformation. The model proposes that DI particles by their interference activity, coded for by S region sequences, directly alter expression of cytotoxic genes and thereby allow L region DNA sequences associated with oncogenesis to be dominantly expressed. Experiments to date confirm this model. We have recently cloned the L region oncogenic DNA sequences as well as S region defective DNA sequences as recombinant DNAs in a plasmid–Escherichia coli system (Robinson et al., 1981) and we are now performing experiments to confirm the role of these virus genes in different biological expressions of this herpesvirus.

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