Characteristics of Parma Wallaby Herpesvirus Grown in Marsupial Cells

By J. MILLAR WHALLEY and CAROL E. WEBBER

School of Biological Sciences, Macquarie University, Sydney, N.S.W., Australia

(Accepted 30 May 1979)

SUMMARY

Parma wallaby herpesvirus (PWHV) has been characterized by a number of properties. Electron microscopic examination of thin sections of infected cell nuclei revealed virus nucleocapsids with various morphologies characteristic of herpesviruses; enveloped particles were seen in cytoplasmic vacuoles and outside cells. Negatively stained virus preparations from the medium of infected cell cultures contained particles with the typical appearance of naked and enveloped herpesviruses. The DNA of PWHV had a mean buoyant density in preparative caesium chloride gradients of 1.712 g/ml, giving an estimated base composition of 51% guanosine plus cytosine. The virus was able to replicate in all marsupial cells, but not in most eutherian cells tested, and a single cycle of infection lasted about 25 h. Infectivity was destroyed by a number of agents including lipid solvents, acid pH and heat. The observed properties support the classification of this virus as a new member of the herpesvirus family.

INTRODUCTION

The isolation of a probable herpesvirus from diseased Parma wallabies (Macropus parma) by Finnie et al. (1976) is the first report of a herpesvirus affecting Australian marsupials. This virus, provisionally referred to as Parma wallaby herpesvirus (PWHV) has been shown to be experimentally transmissible to Parma wallabies, in which it caused a fatal disease similar to that associated with the original isolation (I. R. Littlejohns, E. P. Finnie, H. M. Acland & G. Maynes, personal communication). Evidence that PWHV or a closely related herpesvirus is widespread among Australian marsupials came from a study of virus neutralizing antibody in sera from 242 animals in the wild and 116 in captivity (Webber & Whalley, 1978). Higher levels and frequency of antibody to PWHV were observed among captive animals compared with those in the wild.

The aim of the present study was to characterize PWHV with respect to growth and host range in cell culture, appearance in the electron microscope and buoyant density of virus DNA.

METHODS

Cells and media. Primary cell lines of epithelial morphology were initiated from ear biopsies from M. parma and maintained for approximately 15 passages. A primary cell line of the marsupial mouse Antechinus rosamondae was initiated from body wall tissue by Drs D. W. Cooper and P. Woolley, and maintained for over 30 passages. BHK21 cells were supplied by Commonwealth Serum Laboratories (CSL), Parkville, Victoria, Australia. Canine and feline kidney cells were kindly supplied by Dr M. Sabine, Department of Veterinary Pathology, University of Sydney, Australia. SCB 181 (Wallabia bicolor) cells
were a gift from Dr R. Moore, Cancer Institute, Melbourne, Australia. Other marsupial cell cultures were grown from stocks held by Professor G. B. Sharman and Dr D. W. Cooper. All cells were routinely grown in monolayer cultures in Ham's F10 medium (CSL) containing 10 to 20% (v/v) foetal calf serum (FCS; Australian Laboratory Services or Flow Laboratories) at 35°C in an atmosphere of 5% CO2.

**Virus stocks.** The original stock of PWHV was kindly supplied by Dr I. R. Littlejohns and E. M. Batty, Glenfield Veterinary Research Station, N.S.W., Australia. Herpes simplex type 1 (HSV-1), Bryan strain, was a gift from Dr M. Cloonan, Prince of Wales Hospital, University of New South Wales, Sydney, Australia. Bacteriophage T7 was a gift from Dr M. Sabine. Stocks of PWHV were grown in *M. parma* cells, and HSV-1 in BHK21 cells. all virus stocks were twice plaque purified before use in this study.

**Plaque assay.** PWHV was assayed in 60 mm plastic dishes on confluent monolayer cultures of either *M. parma*, *A. rosamondae* or BHK21 cells. The cells were pre-treated for at least 1 h with 4 μg/ml Polybrene (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), and virus allowed to adsorb for 2 h, before overlaying with medium composed of Ham's F10, 5 to 10% (v/v) FCS and 1.5% (w/v) methylcellulose. Plaques of rounded cells were read microscopically 2 to 4 days p.i. The efficiency of infection in *M. parma* cells was approximately twice that in *A. rosamondae* cells and 100 times that in BHK21 cells.

**Treatment with physical and chemical agents.** Virus samples from culture medium were assayed before and after treatment with formalin, chloroform, ether, acid pH and temperatures of 37 and 56°C (Rouzzo & Burke, 1973), and were also exposed to cycles of freezing and thawing or sonication.

**Virus growth curve.** PWHV stocks were prepared from infected *M. parma* cells by ultrasonication of cells and medium, followed by filtration through a 0.45 μm pore size Millipore filter. Titres of virus prepared in this way were generally between $1 \times 10^7$ and $2 \times 10^7$ p.f.u./ml. *Macropus parma* cells and *A. rosamondae* cells were infected in suspension with PWHV (m.o.i. 36:1) for 1 h in culture medium containing 2 μg/ml Polybrene. Infected cells were washed four times with 6 ml of calcium and magnesium-free phosphate-buffered saline (PBS-) and then plated on to 35 mm diam. plastic dishes in fresh culture medium and incubated at 35°C. At various times p.i. plates were chilled to 0°C, cells scraped off the plates and cells and media separated by centrifugation at 2000 rev/min for 10 min. Cells were washed twice with fresh culture medium and the supernatants pooled and stored at 4°C until infectivity was measured by plaque assay. The cells were resuspended in fresh medium and immediately before plaque assay were sonicated in a Branson sonifier at 50 W for 30 s.

**Electron microscopy.** Infected cells were prepared for electron microscopy by scraping cultures off plastic dishes (when 70% of the cells showed c.p.e.), centrifuging at 2000 rev/min for 5 min, fixing in 2% glutaraldehyde and post-fixing in 1% osmium tetroxide prior to thin sectioning. Virus for negative staining was concentrated from infected cultures by centrifuging clarified media at 270000 g for 1 h and resuspending in a small volume of buffer (0.1 M-NaCl, 0.05 M-tris-HCl, 0.005 M-EDTA, pH 8.0); droplets of virus suspension were picked up on Formvar-coated grids and stained with 2% ammonium molybdate. Preparations were examined either in a Hitachi H8S or a Philips 300 electron microscope.

**Preparation of nucleocapsids.** Nucleocapsids from infected cells were prepared by modification of the method described by Kieff *et al.* 1971. Infected cells were scraped off 100 mm plastic dishes and centrifuged at 800 g for 10 min at 4°C. The cell pellet was washed with cold PBS-, resuspended in RBS (0.01 M-NaCl, 0.001 M-MgCl2, 0.01 M-tris-HCl, pH 7.5) containing 0.5% (v/v) NP40 and left at 4°C for 10 min. The cell suspension was sonicated for 30 s at 50 W and centrifuged at 800 g for 10 min at 4°C. The supernatant was layered on to 10 to 50% sucrose gradients (in 0.15 M-NaCl, 0.02 M-tris-HCl, pH 7.5) and centrifuged
for 30 min at 81 500 \( g \) in a Beckman SW27.1 rotor. Fractions containing nucleocapsids were pooled, pelleted by centrifugation at 270000 \( g \) for 1 h in the SW41 Ti rotor, and allowed to resuspend in 1 M-NaCl, 0.01 M-EDTA, 0.05 M-tris-HCl, pH 7.5, for 16 h at 4 °C. Such preparations consisted mainly (> 80%) of naked particles although some enveloped or partly enveloped particles could also be seen on electron microscopic examination.

**DNA extractions.** PWHV DNA was extracted from nucleocapsids by incubation at 37 °C with 50 \( \mu \)g/ml RNase for 15 min, 30 min with autodigested pronase (50 \( \mu \)g/ml) in 2% Sarkosyl (Ciba-Geigy Australia Ltd., Sydney) and 1% sodium dodecyl sulphate, followed by phenol extraction at 60 °C for 2 min. The aqueous phase was further extracted in chloroform/isooamyl alcohol (50:1) and dialysed against 0.1 M-NaCl, 0.01 M-EDTA, 0.01 M-tris-HCl, pH 7.4. Marker DNA either as DNA (*Micrococcus luteus*) or as whole phage (T7) was added to nucleocapsid preparations and co-extracted with PWHV DNA.

**Radioactive labelling.** Virus DNA was labelled by growing virus in media containing 20 \( \mu \)Ci/ml \(^3\)H-thymidine (The Radiochemical Centre, Amersham). T7 phage DNA was labelled by growing infected *E. coli* B cells in broth containing 5 \( \mu \)Ci/ml \(^32\)P-phosphorus (Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia). The phage was purified by sucrose gradient centrifugation of a polyethylene glycol concentrate ( Hayward & Smith, 1972).

**Analysis of caesium chloride gradients.** DNA preparations were mixed with CsCl in TE buffer (0.05 M-tris-HCl, 0.005 M-EDTA, pH 8.0) to a density of 1.730 g/ml in a vol. of approx. 4 ml and centrifuged in a Beckman 50 Ti rotor for 50 h at 160000 \( g \) at 25 °C. Fractions were collected from the bottom of the gradient through a fine tube and measurements made of refractive index, absorbance and radioactivity. Mean buoyant density values for the DNA were obtained by analysing the data from 12 CsCl gradients, as follows: a regression line was fitted to density versus fraction number for each gradient and each fraction converted to density. Radioactivity was expressed as percentage total radioactivity incorporated into DNA. Each gradient was grouped into 0.002 g/ml density units (for plotting percentage radioactivity against density) and the mean and standard error of the percentage radioactivity for each grouping calculated.

**RESULTS**

**Host range and growth in cell culture**

Cell cultures from a wide range of marsupial species and a number of eutherian species were tested for susceptibility to PWHV as evidenced by the production of c.p.e. in which infected cells typically became rounded up and subsequently detached from the surface of the culture vessel. The virus could also cause plaque formation in permissive cell cultures overlaid with methylcellulose. Little evidence was found for syncytia formation or other forms of cellular aggregation. PWHV was able to grow in cells of all the marsupial species tested (Table I) but only in one of the eutherian cell types, namely the hamster line BHK21, in contrast to the other hamster cell line BOR. In the course of these experiments, some variation was observed in the time taken for c.p.e. (approximately 50% of cells affected) to develop in different cells, for example from about 20 h for wallaby cells up to 120 h for possum cells. The one-step growth cycle of PWHV in *M. parma* and *A. rosamondae* cells was characterized by an eclipse period of between 7 and 10 h, followed by a rapid increase in the titre of infectious virus to a maximum level at about 25 h p.i. Production of virus in the extracellular fraction generally paralleled the rise of virus in the intracellular fraction, although a further increase was observed after 25 h which may have been due to release of virus from dead cells. The overall yield of virus was 262 p.f.u. for each *M. parma* cell and 0.4 p.f.u. for each *A. rosamondae* cell.
Table I. *Host range in tissue culture cells*

<table>
<thead>
<tr>
<th>Permissive cells</th>
<th>Non-permissive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Macropus parma</em> (Parma wallaby)</td>
<td>HeLa</td>
</tr>
<tr>
<td><em>M. eugenii</em> (tammar wallaby)</td>
<td>BSC (African green monkey)</td>
</tr>
<tr>
<td><em>M. rufus</em> (red kangaroo)</td>
<td>Canine</td>
</tr>
<tr>
<td><em>M. giganteus</em> (grey kangaroo)</td>
<td>Feline</td>
</tr>
<tr>
<td><em>M. rufogriseus</em> (redneck wallaby)</td>
<td>BOR (hamster)</td>
</tr>
<tr>
<td><em>M. rufogriseus \times M. agilis</em> (redneck wallaby \times agile wallaby)</td>
<td>XC (rat)</td>
</tr>
<tr>
<td><em>M. r. robustus \times M. rufas</em> (wallaroo \times red kangaroo)</td>
<td><em>Pseudomys novaehollandiae</em> (New Holland mouse)</td>
</tr>
<tr>
<td><em>Macropus robustus erubescens \times M. r. robustus</em> (euro \times wallaroo)</td>
<td><em>Ornithorhynchus anatinus</em> (platypus)</td>
</tr>
<tr>
<td><em>Antechinus rosamondae</em> (marsupial mouse)</td>
<td><em>Rattus fuscipes</em> (native rat)</td>
</tr>
<tr>
<td><em>A. maculatus</em> (marsupial mouse)</td>
<td></td>
</tr>
<tr>
<td><em>Sminthopsis crassicaudatus</em> (marsupial mouse)</td>
<td></td>
</tr>
<tr>
<td><em>Petrogale longmanai</em> (rock wallaby)</td>
<td></td>
</tr>
<tr>
<td><em>P. godmani</em> (rock wallaby)</td>
<td></td>
</tr>
<tr>
<td><em>Thylogale billardierii</em> (Tasmanian pademelon)</td>
<td></td>
</tr>
<tr>
<td><em>Potorous tridactylus</em> (potoroo)</td>
<td></td>
</tr>
<tr>
<td><em>Peramelidae</em> (bandicoot)</td>
<td></td>
</tr>
<tr>
<td><em>Trichosurus vulpecula</em> (brush tailed possum)</td>
<td></td>
</tr>
<tr>
<td><em>Vombaturs ursinus</em> (wombat)</td>
<td></td>
</tr>
<tr>
<td>SCB 181 (swamp wallaby)</td>
<td></td>
</tr>
<tr>
<td>BHK21 (hamster)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Electron micrographs of Parma wallaby ear cells infected with PWHV showing: (a) arrays of nucleocapsids (NC) in cell nucleus; (b) nucleocapsids with various morphologies including particles with dense cores (D), ring-like cores (R) and electron translucent centres (E); (c) cytoplasmic vacuoles (arrow) containing enveloped particles, microtubule-like structures (MT) are also evident; (d) enveloped particle close to the outer cell membrane.
Parma wallaby herpesvirus

Fig. 2. PWHV negatively stained with 2% ammonium molybdate. (a) Non-enveloped capsid and (b) particle with capsid surrounded by distended envelope.

Fig. 3. (a) Separation of particles (mainly nucleocapsids) on 10 to 50% sucrose gradients showing infected (○) and non-infected (●) Parma ear cells. (b) Composite cesium chloride gradient of DNA from nucleocapsid preparation. (Bars show standard errors of mean % radioactivity.) Marker DNA positions are indicated by arrows.

Virus morphology

Infected cells

M. parma cells infected with PWHV were examined in thin sections by electron microscopy. Cell nuclei contained large numbers of non-enveloped particles (nucleocapsids) with a mean diam. of 110 nm, many of which were hexagonal in outline (Fig. 1a, b). Occasional enveloped particles were also seen in cytoplasmic vacuoles (Fig. 1c). The virion shown in Fig. 1(d) clearly possessed 'spikes' projecting from or through an outer membrane surrounding a matrix or ' tegument' (Roizman & Furlong, 1974) which in turn encircles the nucleocapsid. The mean diam. of these 'mature' particles, including the spike region, was 166 nm, with the nucleocapsid structure measuring 110 nm across.
Negatively stained virus

The appearance of negatively stained PWHV is shown in Fig. 2. Under the conditions used most particles appeared to lack an envelope; these ‘naked’ particles appeared hexagonal in outline and some internal structure could be seen in the capsid, including hollow capsomeres (Fig. 2a). An occasional enveloped particle (Fig. 2b) was also seen with a distended envelope surrounding the capsid; surface projections (or spikes) were also visible on part of the envelope.

Buoyant density and base composition of PWHV DNA

DNA was extracted from nucleocapsid preparations (Fig. 3a) and centrifuged to equilibrium in CsCl along with marker DNAs. The results (Fig. 3b) show that PWHV DNA had a mean buoyant density of 1.712 g/ml, corresponding to a base composition of 51% guanine plus cytosine (G+C; Ifft et al. 1961). Under the conditions used, the DNA appeared to be present as two components in approximately equal proportions with buoyant densities of 1.710 g/ml and 1.714 g/ml corresponding to base compositions of 49% and 53% G+C, respectively.

Sensitivity to various treatments

The infectivity of PWHV was readily inactivated by lipid solvents, acid pH, formalin, temperature of 56 °C and by cycles of freezing and thawing. The half-life at 37 °C was less than 1 h.

DISCUSSION

The wide host range exhibited by PWHV in marsupial cells, in contrast to its inability to grow in a number of eutherian cells, suggests that PWHV has evolved along with a marsupial host or hosts. In a previous report, Finnie et al. (1976) found that PWHV grew in Parma wallaby, potoroo and pretty-faced wallaby (M. parryi) cells, but failed to grow in certain bovine, murine or hamster cells. The host range studies on PWHV distinguish it from those herpesviruses which are known to grow in the eutherian cell types tested (Darlington & Granoff, 1973). The growth of PWHV, however, is not necessarily restricted to marsupial cells, as shown by the susceptibility of BHK21 cells to the virus. Although PWHV was unable to replicate in certain human cells, it is of interest that human herpesvirus I (HSV-I) could produce c.p.e. in each of three marsupial cell types tested, namely M. parma, A. rosamondae and a euro×wallaroo hybrid, as well as the Australian native mouse Pseudomys novaehollandiae (unpublished observation).

The type of c.p.e. in cells infected by PWHV was essentially a lytic effect similar to that of many herpesvirus infections in cultured cells (e.g. Darlington & Granoff, 1973). During the original isolation of PWHV, Finnie et al. (1976) described typical eosinophilic intranuclear inclusions and occasional syncytia with giant cell formation. The intracellular growth cycle of 25 h for PWHV in M. parma or A. rosamondae cells compares with 17 h for HSV-I (e.g. Hoggan & Roizman, 1959) and 20 h for some other herpesviruses (Darlington & Granoff, 1973). The yield of PWHV in each M. parma cell of 262 p.f.u. was somewhat lower than reported yields (480 p.f.u.) of HSV-I in cells (Hoggan & Roizman, 1959).

The electron microscopic examination of PWHV-infected cells revealed particles with various morphologies which were characteristic of herpesviruses (reviewed by Watson, 1973, and Roizman & Furlong, 1974) and were consistent with different stages of virus maturation. O’Callaghan & Randall (1976) proposed that partially dense capsids are precursors of the fully dense-cored capsids, which then become enveloped. In the present study, the occurrence in the cytoplasm of PWHV particles with a tegument (Roizman & Furlong, 1974) and an envelope, is in accord with the belief that herpesviruses generally acquire
their outer layers at the cell nuclear membrane (Darlington & Moss, 1969) either by modification of the existing membrane or by virus-induced synthesis of a modified nuclear membrane. The cytoplasmic PWHV particles appeared to be in vacuoles, which may be part of a specific transport mechanism between the nucleus and the outside of the cell (Morgan et al. 1959; Schwartz & Roizman, 1969).

The mean diameters of enveloped PWHV particles (166 nm), nucleocapsids (110 nm), ring-like cores (65 nm) and dense cores (26 nm) are within the range found for other herpesviruses and compare with values for HSV-1 of 120 to 150 nm for enveloped particles, 105 nm for nucleocapsids (Watson, 1973), 45 to 55 nm for ring-like cores and 35 to 45 nm for dense cores (Roizman & Furlong, 1974). The distinctive appearance of negatively stained PWHV particles, especially those with a distended envelope, are highly characteristic of herpesviruses (e.g. Wildy et al. 1966; Watson, 1973).

Although analysis of preparative CsCl gradients does not provide absolute values (such as those obtainable by analytical ultracentrifugation), the buoyant density and base composition of PWHV DNA is in the middle range of values for herpesviruses (Goodheart & Plummer, 1975). Some other herpesviruses with base compositions in this range are cercopithecid herpesvirus 1 and 2 (B virus and SA6) with 51% G+C and herpesvirus saimiri with 50% G+C (from the summary by Roizman & Furlong, 1974). Despite this similarity in base composition of DNA, these viruses are differentiated from PWHV by their host range. The presence of two peaks of DNA in CsCl could be explained by the DNA being extracted in the form of several discrete fragments with different base compositions. The two bands were consistently observed and were unlikely to be due to the presence of two strains of virus as the PWHV stocks were plaque-purified; it is also unlikely that one of the peaks was due to a cellular DNA component, such as a satellite, since the DNA was obtained from nucleocapsid preparations separated on sucrose gradients. When uninfected cells were similarly treated, no material corresponding to the nucleocapsid band was found, also arguing against possible contamination by mycoplasma contributing to the DNA profile. The presence of two peaks of DNA in CsCl gradients has been reported for a number of herpesviruses and has been discussed by Goodheart & Plummer (1975). The DNA of several murine cytomegaloviruses banded at 1.717 g/ml and 1.722 g/ml when present as fragments of one quarter length or less (Plummer et al. 1969; Mosmann & Hudson, 1973).

The data presented here, together with the initial description of clinical symptoms and effects on cultured cells by Finnie et al. (1976), is evidence that this virus from a Parma wallaby is a new member of the herpesvirus family, *Herpetoviridae* (Fenner, 1976) and supports the suggestion (Webber & Whalley, 1978) that PWHV has evolved with a marsupial host, rather than having been recently transferred from another (eutherian) mammalian species.

The authors are grateful to Professor G. B. Sharman, Dr D. W. Cooper and colleagues for access to their stocks of marsupial cells; thanks are also due to Dr D. Cockayne and Mr. R. Wright (Electron Microscope Unit, University of Sydney) and Ms J. Gregory for assistance with electron microscopy; to Ms D. Clucas and Mr R. J. Oldfield for photography. This research was supported by grants from the Australian Research Grants Committee and from Macquarie University Research Grants Committee.

REFERENCES


(Received 5 February 1979)