Characterization of a Tree Shrew Herpesvirus Isolated from a Lymphosarcoma

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SUMMARY

A herpesvirus was isolated from a lymphosarcoma culture of tree shrews (Tupaia belangeri) and termed Tupaia herpesvirus 2 (THV-2). Electron microscopy of THV-2 revealed the presence of virus particles with nucleocapsids of about 100 nm surrounded by large envelopes compatible with virions of the herpesvirus group. An extensive host range study revealed that Tupaia embryonic fibroblasts are the cells of choice for the efficient propagation of THV-2. This cell line was used for the continued propagation and plaque assay of THV-2. The mol. wt. of the virus DNA was found to be 100 × 10^6. The buoyant density of THV-2 was 1.724 g/ml. The DNA of THV-2 was compared to the DNA of herpes simplex virus (HSV) and to another previously isolated herpesvirus from apparently healthy tree shrews (THV-1). The analysis was carried out using the restriction endonuclease EcoRI and the cleavage pattern of THV-2 DNA resulted in DNA fragments which were different from those of HSV-1 DNA and distinguishable from the DNA of THV-1.

INTRODUCTION

A number of herpesviruses have been isolated from different animal species including subprimates and primates (Agrba et al. 1975; Meléndez et al. 1968, 1972; Rasheed et al. 1977). The isolation and electron microscopic characterization of a herpes-like virus THV-1 from a degenerating lung tissue culture from the apparently healthy primitive subprimate Tupaia has been reported previously (Mirkovic et al. 1970; McCombs et al. 1971).

The occurrence of a malignant tumour in tree shrews was first described by Elliot et al. (1966). Our laboratories have been actively involved in investigating the virological aspects of this animal. We recently reported on the activation of an endogenous retrovirus in tree shrews (Flügel et al. 1978) and on the experimental infection of Tupaia with herpes simplex viruses type 1 and 2 (Darai et al. 1977). In parallel to these studies we focussed our attention...
on the possibility of a virus aetiology for neoplasias in tree shrews. Similar investigations with herpesvirus saimiri, herpesvirus ateleis and herpesvirus papio encouraged us to perform these experiments.

This study presents data on the isolation and characterization of a new isolate of a herpesvirus of tree shrew THV-2.

METHODS

Isolation of virus. An adult Tupaia belangeri of approx. 8 years of age developed a rapidly growing proliferating tumour in the mesenterial lymphoid nodes. Two months after this initial observation the moribund animal was sacrificed. The tumour was removed aseptically. The tumour material was divided into several portions and used, first, for the establishment of cell cultures, second, for storage at -70°C, third, for electron microscopy, and fourth, for a histopathological examination. In addition, the serum of the moribund animal was frozen at -70°C for serological studies. Cell cultures from the tumour material were established in tissue culture as described previously (Darai & Munk, 1976) and grown in RPMI 1640 supplemented with 10% foetal calf serum at 37°C. The tumour cell cultures grew rapidly as monolayers. One week later, after the cultures had reached confluency, some cytopathic effect (c.p.e.) with cell degeneration was observed by microscopy. From the supernatants of these cultures, infectious virus was isolated by harvesting and low speed centrifugation of the culture fluids. The virus was provisionally termed herpes virus tree shrew (HVTS; Darai et al. 1978a), but in agreement with the decision of the International Commission for the Taxonomy of Viruses, Herpesvirus Study Group, appointed in 1971, will now be officially termed THV-2.

Cells and media. The following cell cultures were grown in Eagle's basal medium balanced with Earle's salt solution (BME) supplemented with 10% foetal calf serum, twice the standard concentration of amino acids and vitamins, 100 international units penicillin/ml and 100 μg streptomycin/ml (BME-FCS-10): Tupaia embryonic fibroblast cells (TEF-458-2; Darai et al. 1978b), rat embryonic fibroblast cells (REF-1-76; Darai et al. 1977), mouse 3T3 fibroblast cells, vero cells, RC-37 cells, CV-1 cells and hamster embryonic fibroblast cells (HEF). On the other hand the following cell cultures were grown in RPMI 1640 supplemented with 10% foetal calf serum (RPMI-FCS-10): human foreskin fibroblast cells (HFF-3), owl monkey kidney cells and marmoset skin fibroblast cells (HF-3061), kindly provided by Dr F. Deinhardt, München; mink lung cells (MvLu) and dog thymus cells (Fcf2th) supplied by Dr C. Bergholz, Chicago; primary rabbit kidney cells (PRK), primary chick embryo fibroblast cells (CEF) and primary monkey kidney cells (Cercopithecus aethiops).

Plaque assay for THV-2. The plaque assay for THV-2 was carried out according to published procedures (Russell, 1962; Darai & Munk, 1976) and modified as follows: TEF-458-2 cell cultures were freshly trypsinized, seeded into Linbro plastic plates (6 x 4 wells, 16 mm diam., each well containing 5 x 10⁴ cells) and incubated in a 5% CO₂-air atmosphere at 37°C for 24 h prior to infection. Serial 10-fold dilutions of each virus sample were made in BME without FCS and 0.1 ml of each dilution was inoculated into four wells of a Linbro plastic plate containing confluent monolayers of TEF-458-2 cell cultures. After 1 h of absorption at 37°C with shaking every 15 min, the cultures were overlaid with 0.5 ml/well of BME-FCS-10 containing 0.5% carboxymethylcellulose. The cultures were maintained for 6 days at 37°C in a 5% CO₂-air atmosphere. Thereafter the cultures were fixed with 5% formaldehyde, stained with 1% crystal violet and the number of virus plaques counted.

Preparation of virus stocks. THV-2 was propagated on TEF-458-2 which had been infected at a multiplicity of infectious virus (m.o.i.) of 0.01 p.f.u./cell and incubated at 37°C. On day 5 p.i., at maximum c.p.e., culture fluids were collected, centrifuged at 800 g to remove cell
debris and the supernatants were frozen at −70 °C until use. The virus titre was determined by the plaque assay as described above. The TS-23 isolate of *Tupaia* herpesvirus 1 (McCormick *et al.*, 1971), supplied by Dr J. Melnick, Baylor College of Medicine, Houston, was also propagated in TEF-458-2 cell cultures, harvested and assayed by the plaque method described above.

**Electron microscopy.** Cell monolayers were fixed in situ by the addition of glutaraldehyde to the tissue culture medium to a final concentration of 2.5%. After 20 min at 37 °C the cells were removed from the plastic dishes using a rubber policeman, centrifuged and post-fixed with 1.0% osmium tetroxide. The cell pellets were block-embedded by agar enclosure, treated for 1 h with 1% uranyl acetate, dehydrated and embedded in Epon according to published procedures (Gelderblom *et al.*, 1974, 1978). Ultrathin sections were cut on a Reichert OmU3 type ultramicrotome, post-stained with lead citrate (Venable & Coggeshall, 1965) and screened in a Siemens type 101 electron microscope. In addition, pellets from centrifuged tissue culture supernatants and virus fractions purified by gradient centrifugation were examined after negative staining.

**Velocity sedimentation analysis of virus particles.** THV-2 particles labelled with 3H-thymidine as described below were pelleted by centrifugation at 20,000 rev/min in a SW27 rotor at 4 °C for 45 min. Subsequently, virus particles were resuspended in 0.15 M-NaCl, 0.02 M-tris-HCl, pH 7.2 (virus buffer) and layered on 20 to 50% sucrose (w/v) gradients in virus buffer and centrifuged at 20,000 rev/min in a SW27 rotor at 4 °C for 20 min. Fractions were collected, precipitated with 5% TCA (Darai *et al.*, 1975) and virus particles were monitored in gradient fractions by measuring the radioactivity of the incorporated 3H-thymidine.

**Preparation of labelled virions and virus DNA.** Monolayers of TEF-458-2 cell cultures were infected with THV-2 at a m.o.i. of 0.01 and incubated for 1 h at 37 °C. Afterwards the cultures were washed five times with BME without FCS, re-fed with BME-FCS-10 and incubated at 37 °C. 3H-thymidine (1 μCi/ml, sp. act. 48 Ci/mmol) was added to the cultures 24 h p.i. Labelled THV-2 and labelled TS-23 virus (THV-1) were isolated 5 days p.i. as described above. Virus DNA was prepared from virus as previously described (Darai *et al.*, 1975) and analysed by isopycnic gradient sedimentation in CsCl. Briefly, extracted DNA was banded in CsCl equilibrium density gradients with an initial density of 1.700 g/ml in a Ti50 Spinco fixed angle rotor for 48 h at 38,000 rev/min at 20 °C.

**Velocity sedimentation analysis of THV-2.** The sedimentation rate of the isolated virus DNA was determined in 10 to 30% sucrose (w/v) gradients containing 10 mm-trypt-HCl (pH 7.8), 1 mm-EDTA and 0.15 M-NaCl in a Spinco SW40 rotor for 3.5 h at 40,000 rev/min. DNA of herpes simplex virus (HSV) type 1, strain ANG (Schröder *et al.*, 1975/76) served as a sedimentation marker (60 ± 1S, corresponding to a mol. wt. of 100 × 106).

**Restriction endonuclease cleavage and gel electrophoresis.** Virus DNA was digested with the restriction endonuclease EcoRI and the resulting fragments were separated on a 0.5% agarose slab gel. Electrophoresis was performed in 0.5% agarose vertical gels (up to a size of 60 by 20 by 0.3 cm) as described by Thomas & Davis (1975). The gels were stained after electrophoresis with ethidium bromide (0.5 μg/ml) and photographed under u.v. light.

**Neutralization test.** Neutralization tests were carried out by diluting rabbit anti-THV-2 sera with PBS (1:2, 1:4 to 1:256) in a Falcon microtitre plate. Serum dilutions (50 μl) were mixed with 100 TCID50 of THV-2 per 50 μl. The serum–virus mixture was incubated in a 5% CO2-air atmosphere at 37 °C for 2 h. Subsequently, 5 × 103 TEF-458-2 cells in suspensions were added to each sample of the serum-virus mixture. After 12 h the infected cultures were overlaid with BME-FCS-10 containing 0.5% carboxymethylcellulose and then incubated at 37 °C in a 5% CO2-air atmosphere for 6 days. Subsequently, the cultures were stained with 1% crystal violet and the titres were determined.
Antiserum. A suspension of 2 ml of THV-2 purified by sucrose gradient centrifugation, containing $5 \times 10^6$ p.f.u./ml, was inoculated intramuscularly into adult white New Zealand rabbits. Inoculation was repeated weekly for 4 weeks and an additional inoculation was given intravenously after 5 weeks. One week later, the rabbits were bled and the resulting anti-THV-2 sera was frozen at $-70$ °C until use. Goat antiserum against herpesvirus saimiri was kindly provided by Dr F. Deinhardt, München. Rabbit antiserum against HSV-1 Thea and against HSV-2 Müller were prepared as described previously (Darai & Munk, 1976).

RESULTS

Lymphosarcoma culture

A lymphosarcoma of an 8-year-old tree shrew was established in tissue culture. The cultures grew rapidly in an undisturbed and fibroblastic manner and then degenerated with c.p.e. typical of a cytoidal viral infection (Fig. 1).

Morphology of THV-2

The degenerating cell cultures were screened for the presence of virus particles by electron microscopy at the first passage. Most of the cells contained either intra- or extracellular virus-specific structures. In the nuclei, naked virus capsids with a diam. of about 100 nm were observed which contained cores of varying density. Extracellular virus particles had an additional envelope studded with small surface projections, which were hardly visible in thin sections. Beneath this limiting membrane, a remarkably electron-dense zone of high granularity was found. Some of the envelopes contained several virus capsids. The diameter of the envelope ranged from 200 to 350 nm (Fig. 2). Negative staining of the pellets of the
Fig. 2. Ultrathin section of a *Tupaia* embryonic fibroblast cell 5 days after infection with tree shrew herpesvirus. The nucleus has three virus capsids and the mature virion in the extracellular space contains two capsids.
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Fig. 3. Negative staining of pellets of tissue culture supernatants using 2% phosphotungstic acid at pH 7.2 reveals the relatively high purity of such materials. Envelope structures are remarkably stable and contain different numbers of capsids.

Table I. Host range of herpesvirus tree shrew (THV-2): all cell cultures were inoculated at a density of $1 \times 10^6$ cells with an m.o.i. of 0.1 p.f.u./cell

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Susceptibility to THV-2</th>
<th>Resulting virus titre (p.f.u./ml)</th>
<th>Virus yield (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tupaia embryonic fibroblast (TEF-458-2)</td>
<td>+</td>
<td>1-2/6-8</td>
<td>$2.5 \times 10^6-1.5 \times 10^7$</td>
</tr>
<tr>
<td>Primary rabbit kidney cells (PRK)</td>
<td>+</td>
<td>3-4/7-9</td>
<td>$5.2 \times 10^5-6.8 \times 10^5$</td>
</tr>
<tr>
<td>Human foreskin fibroblast (HFF-3)</td>
<td>+</td>
<td>7-8/25-30</td>
<td>$2.5 \times 10^8-2.8 \times 10^8$</td>
</tr>
<tr>
<td>Marmoset skin fibroblast (HF-3061)</td>
<td>+</td>
<td>2-6/30-35</td>
<td>$1.3 \times 10^5-2.1 \times 10^5$</td>
</tr>
<tr>
<td>Mink lung (MvILu)</td>
<td>+</td>
<td>15-16†</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Other‡</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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</table>

* Only proliferative c.p.e. without plaque formation.
† No lysis during 2 months observation period.
‡ Primary monkey kidney cells (Cercopithecus aethiops), African green monkey kidney cells (Vero, CV-1), monkey kidney cells (RC-37), dog thymus cells (Frfzth), rat embryonic fibroblasts (REF-1-76), hamster embryonic fibroblasts (HEF), mouse embryonic fibroblasts (3T3) and chick embryonic fibroblasts (CEF).

Tissue culture fluids revealed particles with similar features. The envelopes were only poorly penetrated by the stain regardless of diameter and contained different numbers of virus capsids (Fig. 3). According to these morphological data this isolate can be assigned to the herpervirus group. In order to characterize this virus further, a study of its host range was made.

Host range determination

A variety of different cell cultures listed in Methods were inoculated with purified supernatants of the original tumour cell cultures at a m.o.i. of 0.1 p.f.u./cell. The results are given in Table I. In TEF-458-2 cells, c.p.e. was first observed 48 h after inoculation and progressed rapidly to a complete degeneration of monolayers within 8 days with concomitant production of floating cells. In contrast, all other cell cultures except PRK, HFF-3, HF-3061 and MvILu cells showed no c.p.e. during a 6-week observation period. The development of...
c.p.e. in HFF-3 was slow and was first observed after 1 week with plaque formation and progressed during the next 3 weeks to complete degeneration. In the case of Mv1Lu cells, c.p.e. was first observed with plaque formation but did not progress in the following 6 weeks. This host range indicates that the observed c.p.e. in the original tumour cultures was not caused by a contaminating herpes simplex virus, the host range of which is completely different. We also concluded from the host range of THV-2 that the cells of choice for its propagation were TEF-458-2 cells.

Plaque assay and virus growth kinetics

The rapid degeneration of TEF-458-2 cell cultures with THV-2 led to development of a plaque assay for THV-2. To determine the infectivity of THV-2 particles, assays were carried out as described in detail in Methods. The plaques were of a clear and distinct appearance. To determine the growth kinetics of THV-2, monolayers of TEF-458-2 cell cultures were infected with a m.o.i. of 0.01 p.f.u./cell, incubated at 37°C and the infectivity titre of the culture fluids determined daily by a plaque assay. Fig. 4 shows that the maximal infectivity (1 x 10⁷ p.f.u./ml) was reached 5 days after infection. The resulting virus growth kinetics demonstrates that with a low m.o.i. of 0.01 p.f.u./cell, a high virus yield can be reached thus facilitating further studies with THV-2.

Velocity sedimentation of virions

The sedimentation rate of ³H-thymidine labelled THV-2 particles was greater than that of ¹⁴C-thymidine labelled HSV-2 virions (Fig. 5). For the further classification of THV-2, experiments were performed to determine the buoyant density and the mol. wt. of THV-2 DNA.
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Fig. 6. Isopycnic sedimentation of herpesvirus tree shrew (THV-2) DNA in CsCl. •—• ³H-HSV-1 ANG marker + ³H-SV40 DNA; ○—○, ¹⁴C-THV-2 DNA. Further details in Methods.

Fig. 7. Velocity sedimentation of tree shrew herpesvirus DNA. A sample of ³H-thymidine labelled DNA was sedimented through a 10 to 30 % sucrose gradient. ¹⁴C-labelled, HSV-1 ANG DNA (6) served as a sedimentation marker (6o ± 1S). ■—■ ³H-THV-2 DNA; ○—○, ¹⁴C-HSV-1 DNA.

Analysis of virus DNA

Determination of buoyant density

The ³H-labelled THV-2 DNA was prepared from TEF-458-2 cell cultures infected with THV-2 and labelled with ³H-thymidine. After purification of the THV-2 particles by low and high speed centrifugation, virus DNA was extracted according to the method described previously (Darai et al. 1975). The buoyant density of THV-2 DNA was found to be ρ = 1.724 g/ml (Fig. 6). To determine the difference in the buoyant densities of THV-1 (Ludwig, 1972) and THV-2 DNA, the ³H-labelled THV-2 DNA and ¹⁴C-labelled THV-1 DNA were analysed in the same CsCl gradient. It was found that under identical conditions the two peaks differ by one fraction from one another, the buoyant density of THV-1 DNA being practically identical to that of THV-2 DNA (data not shown.)

Molecular weight determination of THV-2 DNA

³H-labelled THV-2 DNA isolated from purified virus particles co-sedimented as a single sharp band with HSV-1 (ANG) DNA (Fig. 7). The mol. wt. of THV-2 DNA was shown to be 100 x 10⁶, similar to HSV DNA (Russell & Crawford, 1964; Kieff et al. 1971).

Restriction endonuclease cleavage pattern of THV-2 DNA in comparison to HSV and THV-1 DNA

The analysis of THV-2 DNA by the restriction endonuclease EcoRI shows a characteristic fragment pattern of ten DNA bands. HSV DNA strain ANG (Schröder et al. 1975/76; Darai & Munk, 1976) was also cleaved by EcoRI and run on the same gel as a control (data not shown). It was found that the DNA of THV-2 had a different cleavage pattern from HSV DNA. In order to compare THV-2 DNA with THV-1 DNA, a digestion of both DNA strains with the restriction endonuclease EcoRI were performed separately and subsequently run on an agarose gel. The resulting cleavage pattern presented in Fig. 8 shows that the two
Fig. 8. Cleavage of tree shrew herpesvirus (THV-2) DNA and of *Tupaia* herpesvirus THV-1 DNA by restriction endonuclease *EcoRI*. The DNAs were separated on a 0.5% agarose slab gel. (A) Lambda DNA digested with *EcoRI* served as a marker. The mol. wt. of the individual bands (from top to bottom) are 31.0, 15.8, 13.7, 4.7, 3.7, 3.5, 3.0 and 2.1 × 10⁶. (B) THV-2 DNA digested with *EcoRI*. The mol. wt. of the individual bands (from top to bottom) are 16.5 to 19.2, 12.6, 9.4, 7.4, 7.0, 5.1, 4.4, 3.7, 1.7 × 10⁶. (C) THV-1 DNA digested with *EcoRI*. The mol. wt. of the individual bands (from top to bottom) are 22.5, 16.5 to 19.2, 12.6, 9.4, 8.8, 7.4, 5.1, 3.7, 3.1, 1.5 × 10⁶.

Table 2. *Neutralization capacity of rabbit anti-THV-2 serum against THV-1 and THV-2*

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>Neutralization titre</th>
</tr>
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<tbody>
<tr>
<td>THV-2</td>
<td>1:1024</td>
</tr>
<tr>
<td>THV-1</td>
<td>1:256</td>
</tr>
<tr>
<td>HSV-1 strain (Thea)</td>
<td>&lt; 1:10</td>
</tr>
<tr>
<td>HSV-2 strain (Müller)</td>
<td>&lt; 1:10</td>
</tr>
</tbody>
</table>

Virus strains are different from each other, although the cleavage pattern indicates that most of the DNA fragments of the tree shrew viruses co-migrate. The data presented lead us to suggest that this THV isolate is a new and distinct strain of herpesvirus, which we have named THV-2.
Neutralization test

The serological characterization of THV-2, which was performed as described in Methods, showed that rabbit antisera against THV-2 did not neutralize HSV-1, HSV-2 or herpesvirus saimiri, but neutralized the THV-1 isolate. Although the rabbit anti-THV-2 sera cross-reacted with THV-1, it was possible to distinguish THV-1 and THV-2 in neutralization tests, by significant differences in the resulting titres (Table 2). Under the same test conditions, the neutralization capacity of rabbit anti-THV-2 sera was found to be four dilution steps lower when THV-1 was used.

DISCUSSION

Our data reported here demonstrate that the herpesvirus THV-2, which was isolated from a lymphosarcoma of a tree shrew, is different from known herpesviruses as judged by its host range and its characteristic cleavage pattern by the restriction endonuclease EcoRI. The morphology of the THV-2 virus isolate is similar to that of THV-1 (McCombs et al. 1971) and fits that of the herpesvirus group well. The THV-2 virion consists of an envelope of varying diameter containing the virus capsid of 100 nm diam. THV-2 can be differentiated from herpes simplex viruses by its much more stable envelope and by the very dense granular zone beneath this membrane structure. However, morphologically THV-2 closely resembles the other tree shrew herpesvirus isolate THV-1. Nevertheless, THV-2 is different from the THV-1 isolate in its host range and its characteristic cleavage pattern. EcoRI cleaved THV-2 DNA into ten distinguishable DNA fragments and THV-1 DNA into eleven fragments (Fig. 8). Four of the THV-2 DNA fragments appeared not to be present in the cleavage pattern of THV-1 DNA. Concerning the host range, THV-2 grows on marmoset skin fibroblast cells but THV-1 does not (Mirkovic et al. 1970). The reported data do not rule out the possibility that THV-1 could be propagated on marmoset skin fibroblast cells after successful adaptation to this host cell.

THV-2 shows some properties which are typical for other herpesviruses, e.g. the structure of its virions and the mol. wt. of $100 \times 10^6$ for its DNA. The investigation of the pathogenesis and oncogenesis of THV-2 is the subject of further studies which are in progress.

Note added. Recently a third herpesvirus (THV-3) was isolated from a lymphosarcoma of another tree shrew also imported from Thailand. The EcoRI cleavage pattern of THV-3 DNA was found to be similar but clearly different from either the fragment pattern of THV-1 and of THV-2 DNA. Recent determinations of buoyant densities of DNAs of the three THV strains by analytical ultracentrifugation revealed that they have an identical G+C content of 65.4%.

We are especially grateful to Professor Dr Wilhelm Doerr, Institute of Pathology, University of Heidelberg for the histo-pathological diagnosis of the lymphosarcoma. We thank Dr J. L. Melnick for providing the TS-23 virus isolate (THV-1) and Dr A. Schwaier for supplying the lymphosarcoma. We thank Miss Ulrike Gramlich and Mr H. Reupke for their excellent technical assistance.
REFERENCES


MELÉNDEZ, L. V., DANIEL, M. D., HUNT, R. D. & GARCÍA, F. G. (1968). An apparently new herpesvirus from primary kidney cultures of squirrel monkey (Saimiri sciureus). Laboratory Animal Care 18, 374-381.


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