

The Isolation and Partial Characterization of a Highly Pathogenic Herpesvirus from the Harbor Seal (*Phoca vitulina*)

By

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With 4 Figures

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Summary

This report describes the first isolation and partial characterization of a herpesvirus from the harbor seal (*Phoca vitulina*). The virus was isolated during a disease outbreak in a group of young seals nursed in a seal orphanage in The Netherlands. Almost half of the seals died with symptoms of acute pneumonia and focal hepatitis and the virus was isolated of organs of most of the dead animals. Seven out of ten seals of which paired serum samples were obtained showed seroconversion in a virus neutralization test during this outbreak. The virus was tentatively characterized as a herpesvirus (seal herpesvirus: SeHV or phocid herpesvirus 1) on the basis of its characteristic morphology in electron microscopy, buoyant density in sucrose, sensitivity to ether and heat treatment and its antigenic relationship with other probable members of the Alphaherpesvirinae subfamily. The virus caused cytopathic changes within 24 hours after inoculation in seal kidney cells, consisting of a focal rounding of cells and syncytium formation. No cytopathic changes were observed in the cells of nine other mammalian species tested.

Introduction

Herpesviruses have been observed in hosts as diverse as a fungus, oyster, fish, amphibians, reptiles, birds and numerous mammals (4). The family Herpesviridae is divided in three subfamilies: Alpha-, Beta- and Gammaherpesvirinae. Members of all three subfamilies may cause serious illness in mammalian hosts (for review see 7). In the present paper we describe

the first isolation and partial characterization of a herpesvirus from the harbor seal (*Phoca vitulina*): seal herpesvirus (SeHV) or phocid herpesvirus 1. Infection with this virus was associated with serious disease symptoms of acute pneumonia and general depression in young harbor seals nursed in a specialized seal orphanage in The Netherlands. Eleven out of the 23 animals, which all developed clinical illness, died during this period which lasted for about four weeks. Upon post-mortem examination the most striking features were acute pneumonia and focal hepatitis in all the animals examined. We demonstrated that SeHV is a probable new member of the Alphaherpesvirinae subfamily, antigenically related to both canine herpesvirus (CHV) and feline viral rhinotracheitis virus (FVRV).

Materials and Methods

Animals and Sampling Procedures

A group of 23 harbor seals from the Waddenzee (The Netherlands), between 1 and 6 weeks of age, was nursed in a specialized seal orphanage in Pieterburen in The Netherlands. From nine of the animals which died during the disease outbreak, 10 per cent (w/w) suspension of lungs and livers in Dulbecco's modified Eagle's medium (DMEM) were prepared and stored at -70°C until used in virus isolation procedures. Serum samples were collected from the seals by venous puncture seven days and 20 days after the first seal had died ("pre" and "post" serum respectively) and stored at -20°C .

Seal Kidney Cell Cultures

Seal kidney monolayer cell cultures were prepared by standard trypsinization methods, from kidneys of two dead harbor seals, one of which had died in the animal orphanage during the outbreak, the other of which was found dead at one of the Dutch islands in the Waddenzee (SeKC-1 and SeKC-2 respectively). Cells were seeded into 75 cm² plastic tissue culture bottles (Costar, Cambridge, Mass., U.S.A.) at a density of 3×10^5 cells/ml in F-10 maintenance medium (Gibco, Paisley, Scotland), supplemented with 10 per cent FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. For virus isolation SeKC-2 cells were subcultured in Leighton tubes and for growth-curve experiments and infectivity titrations in 25 cm² plastic tissue culture bottles (Costar) or microtiter plates (Costar).

Virus Isolation Procedures

Apart from the virus isolation in the SeKC-1 culture, lungs and livers were selected as organs for attempted virus isolation. The organ suspensions (0.1 ml) were inoculated onto SeKC-2 Leighton tube cultures. After incubation for 2 hours at 37°C the medium was refreshed and the cultures were incubated at 37°C and checked daily for cytopathic changes. After development of cytopathic changes, the culture medium was collected and coverslips were washed with PBS and fixed with ethanol at -70°C , or with formaldehyde at room temperature for indirect immunofluorescence (IF) or haematoxylin and eosin (HE) staining respectively.

Indirect Immunofluorescence Technique

On ethanol fixed (-70°C) coverslips from Leighton tube cultures 1:30 diluted seal serum in phosphate buffered saline (PBS) was applied. After incubation for

15 minutes in a moist chamber at room temperature, the slides were washed in two changes of PBS and two changes of distilled water, for a total of 30 minutes. Then a 1:30 diluted protein-A-FITC conjugate (Pharmacia, Uppsala, Sweden) was applied and the same incubation and washing procedures were carried out. The slides were dried, mounted in glycerin and examined with an epifluorescence microscope as previously described (6).

Virus Growth Cycle in SeKC-2 Cells

Confluent SeKC-2 75 cm² monolayer cultures were trypsinized and 4×10^7 cells were incubated with 10^4 TCID₅₀ SeHV (SeKC-1 supernatant fluid) in 10 ml F-10 maintenance medium. The suspension was incubated for two hours at 37° C in a tube placed in a roller bottle, rolling at about 30 rev/min. The cells were washed twice, suspended in 50 ml maintenance medium, supplemented with 10 per cent FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin, divided amongst ten 25 cm² plastic tissue culture bottles (Costar), and incubated at 37° C. At various time intervals bottles were removed, and 1 ml samples of the culture medium were taken. The remainder of the medium was decanted, and after washing the cells twice replaced by 3 ml of fresh medium. The whole bottles were then frozen and thawed once before a second 1 ml sample was taken. These samples were taken to represent infectious virus present in the medium and cell-associated virus respectively. Infectivity of the samples was assayed in microtitre plates (see below).

Electron Microscopy

For negative contrast examination, small drops of SeKC-1 cell sediment samples after cell lysis by osmotic shock were placed on formvar/carbon-coated grids, and contrasted with 2 per cent phosphotungstic acid (adjusted to pH 5.2 with KOH).

For thin section examination, trypsinized SeKC-1 cells infected with SeHV were fixed in 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 with 0.1 M sucrose added for 12 hours at 4° C, embedded in agar, postfixed in 2 per cent OsO₄ + 1.5 per cent K₄Fe(CN)₆ in the same buffer for 1 hour at 4° C, dehydrated and stained en-bloc with uranylacetate in a graded ethanol-series, and embedded in epoxy resin (glycidether 100, Merck, Darmstadt, FRG). Thin sections were prepared and stained routinely with uranylacetate followed by leadcitrate. The preparations were examined in a Philips EM 400 T transmission electron microscope.

Ether Sensitivity Test

Diethylether (Merck, Darmstadt, FRG) 0.2 ml was added to 0.8 ml of virus suspension and thoroughly mixed by shaking. The mixture was then placed at 4° C for 10 minutes. The aqueous phase was aspirated and tested for infectivity as described below. As controls the titre reductions of herpes simplex virus 1 and Reovirus 3 for Vero cells (see below) were determined to be more than 5.9 log₁₀ and less than 0.5 log₁₀ TCID₅₀ respectively.

Buoyant Density in Sucrose

Virus buoyant density in sucrose was determined by isodensity gradient centrifugation at 4° C. One ml virus suspension (culture supernatant fluid) was layered on top of a linear 10–50 per cent (w/w) sucrose gradient. After centrifugation for 4 hours at 35,000 rpm in a Beckman SW 41 rotor, the gradient was fractionated by bottom unloading; infectivity of the fractions was measured in the infectivity test described below and buoyant density of the fractions was calculated after measuring their refractive indices (2, 8).

Infectivity and Neutralization Tests

For infectivity titrations ten-fold dilutions of samples were made in F-10 maintenance medium, supplemented with 10 per cent FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 100 µl volumes were inoculated in microtiter tissue culture plates (Costar). Then 3×10^4 cells in 100 µl of the same medium were added per well, the plates were sealed and incubated for four days at 37° C in a moist 5 per cent CO₂ atmosphere. The results of these infectivity titrations were read microscopically.

For virus neutralization (VN) assays, sera were heat inactivated for 30 minutes at 56° C and diluted in a two-fold series in tissue culture microtiter plates (Costar). The serum dilutions (50 µl) were incubated with 50–100 TCID₅₀ virus (50 µl) for 2 hours at 20° C. Subsequently 3×10^4 freshly trypsinized cells in 50 µl maintenance medium were added per well and the plates were further processed as described for infectivity titrations. The cells used for the different viruses in both assays are listed below.

Viruses and Antisera

Herpes simplex virus type 1 (HSV-1) strain 63-3390 was kindly provided by Dr. J. G. Kapsenberg of this Institute. It was propagated in an African green monkey kidney cell line (Vero cells) and culture supernatant fluids were used as virus preparations.

Canine herpesvirus (CHV) strain F 205, obtained from Dr. Wright (Department of Veterinary Pathology, University of Glasgow, Scotland), was propagated in primary dog kidney cells or in a continuous dog kidney cell line (MDCK) as previously described (5). Culture supernatant fluids were used as virus preparations.

Feline herpesvirus-1 (FVRV) strain B 927 was kindly provided by Dr. R. Gaskell and propagated in a feline embryo (FE₁) cell line, established in our laboratory. Culture supernatant fluids were used as virus preparations.

Reference antiserum preparations against established members of the Herpesviridae family were obtained from different sources. Antisera against herpes simplex viruses 1 and 2 were obtained from Dr. J. G. Kapsenberg of our Institute. Antisera against bovine mamillitis virus, pseudorabiesvirus, equine rhinopneumonitis virus and equine coital exanthema virus were obtained from Dr. B. Klingeborn (State Veterinary Institute, Biomedical Centre, Uppsala, Sweden). Antisera against Marek's disease herpesvirus and turkey herpesvirus were obtained from Dr. R. M. S. Wira-hadiredja (Central Veterinary Institute, Lelystad, The Netherlands). All these antisera are routinely used in virus neutralization tests as reference preparations for virus identification and have considerable neutralizing antibody titers in homologous tests according to the suppliers. Canine antisera with and without antibodies against CHV were obtained from SPF dogs (Centraal Proefdieren Bedrijf TNO, Zeist, The Netherlands) free from all other known canine viruses, as was shown by routine serologic screening procedures (OSTERHAUS, unpublished observation). Some of these dogs were infected with CHV. Feline antisera against FVRV were obtained from household cats which were screened in a VN test for the presence of neutralizing anti-FVRV antibodies.

Infection of Cells from Different Animal Species

Confluent 75 cm² monolayer cultures of the following cells grown in DMEM with 10 per cent FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin were inoculated with the SeHV isolate using standard procedures: Vero cells (African green monkey

kidney cell line); tertiary cynomolgus monkey kidney cells; HEp-2 (human epidermoid larynx carcinoma cell line with HeLa markers); PK 15 (porcine kidney cell line); primary dog kidney cells; MDCK (canine kidney cell line); FE₁ (feline embryo cell line); primary rabbit kidney cells; RK₁₃ (rabbit kidney cell line); Ratec (rat embryo cell line) and BHK (baby hamster kidney cell line). Similarly CHV and FVRV were inoculated onto the seal, dog and cat cells.

Results

Virus Isolation

Primary harbor seal kidney cell monolayer cultures were prepared from two young animals in 75 cm² plastic tissue culture bottles. The first of these seals had died in the seal orphanage with symptoms of acute pneumonia and focal hepatitis as was confirmed by post mortem examination (Dr. G. BORST, personal communication). The second seal had been found dead at one of the Dutch islands in the Waddenzee and did not show any post mortem indications for infectious disease. In uninoculated cultures of the first seal (SeKC-1), cytopathic changes suggestive for a herpesvirus infection developed spontaneously by the third day. They consisted of a focal rounding of cells and syncytium formation, progressing to involve the entire monolayer by the seventh day (Fig. 1a and b). Primary and subsequent SeKC subcultures of the second seal (SeKC-2) did not show any cytopathic changes and these were used for further passage of the agent causing the cytopathic effect: filtered (220 nm pore filters) SeKC-1 culture medium samples (0.2 ml) inoculated onto SeKC-2 in Leighton tube cultures caused the same cytopathic changes in these cultures within 24 hours. Further passages both with culture medium and infected cells were performed in SeKC-2 cultures. Liver and/or lung suspensions from 9 seals which had died during the disease outbreak were also inoculated onto SeKC-2 Leighton tube cultures. The same cytopathic changes were observed in cultures inoculated with materials from all 9 seals including 8 cultures inoculated with lung suspensions and 3 cultures inoculated with liver suspensions. All these monolayers were tested after -70° C ethanol fixation in an indirect IF test with the "pre" and the "post" serum of seal 84-23, which survived the outbreak, using a protein A-FITC conjugate. In all cultures showing cytopathic changes immunofluorescence was observed with the "post" seal serum and not with the "pre" serum at places coinciding with cytopathic changes. This was shown by HE staining of -70° C ethanol fixed coverslips which had first been used for IF testing (Fig. 1c and d). No immunofluorescence was demonstrated with "pre" or "post" serum in cultures which did not exhibit cytopathic changes. Bacteriological examination of the organs was negative in most cases and no consistent involvement of any microorganism could be demonstrated (Dr. G. BORST, personal communication).

Electron Microscopy

Enveloped as well as naked herpes types virus particles were revealed in negative contrast preparations of cell sediments of disrupted SeKC-1 cells. Viral capsids, measuring approximately 115×105 nm at occasions clearly showed the hollow capsomeres characteristic of herpes viruses (Fig. 2a, b

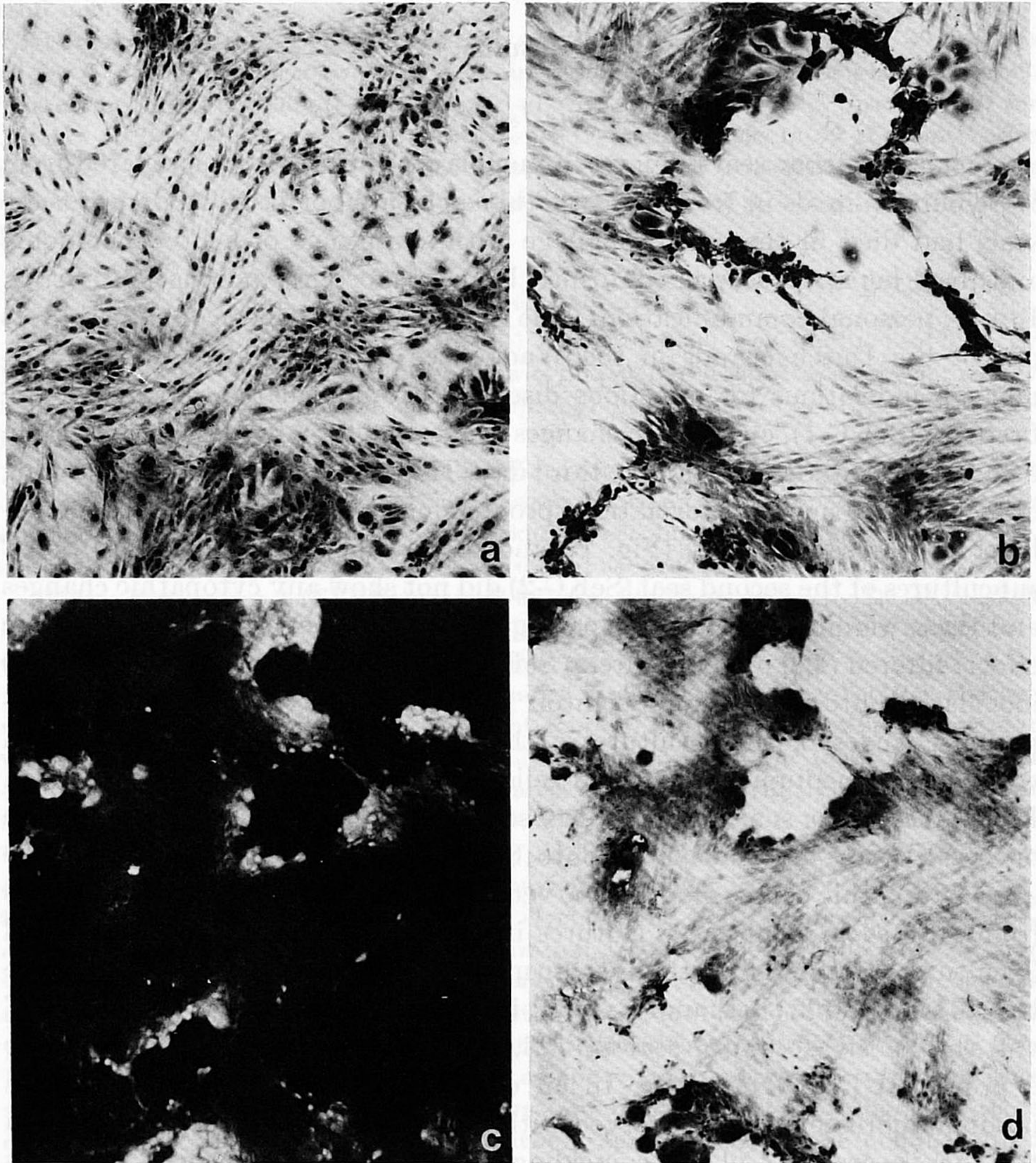


Fig. 1. SeKC-2 Leighton tube cultures: *a* Uninfected, HE stained; *b* SeHV infected, 48 hours after infection with supernatant fluid of SeKC-1 culture, HE stained: foci of infected cells; *c* SeHV infected, 48 hours after infection with supernatant fluid of SeKC-1 culture, fixed with ethanol at -70°C and fluorescent antibody staining with day 20 seal serum 84-23 and protein-A-FITC: fluorescence in foci of infected cells; *d* same as *c* but stained with HE after immunofluorescence testing: same foci of infected cells as in *c*

and c). Thin sections of epoxy resin-embedded SeKC-1 cells, in addition to prominent nuclear and cytoplasmic changes, displayed mainly intranuclear virus particles, i.e. both empty and core-containing viral capsids with occasional hexagonal outline, and some enveloped viral capsids inside the perinuclear space, both obviously reminiscent of herpes virus (Fig. 2 d and e).

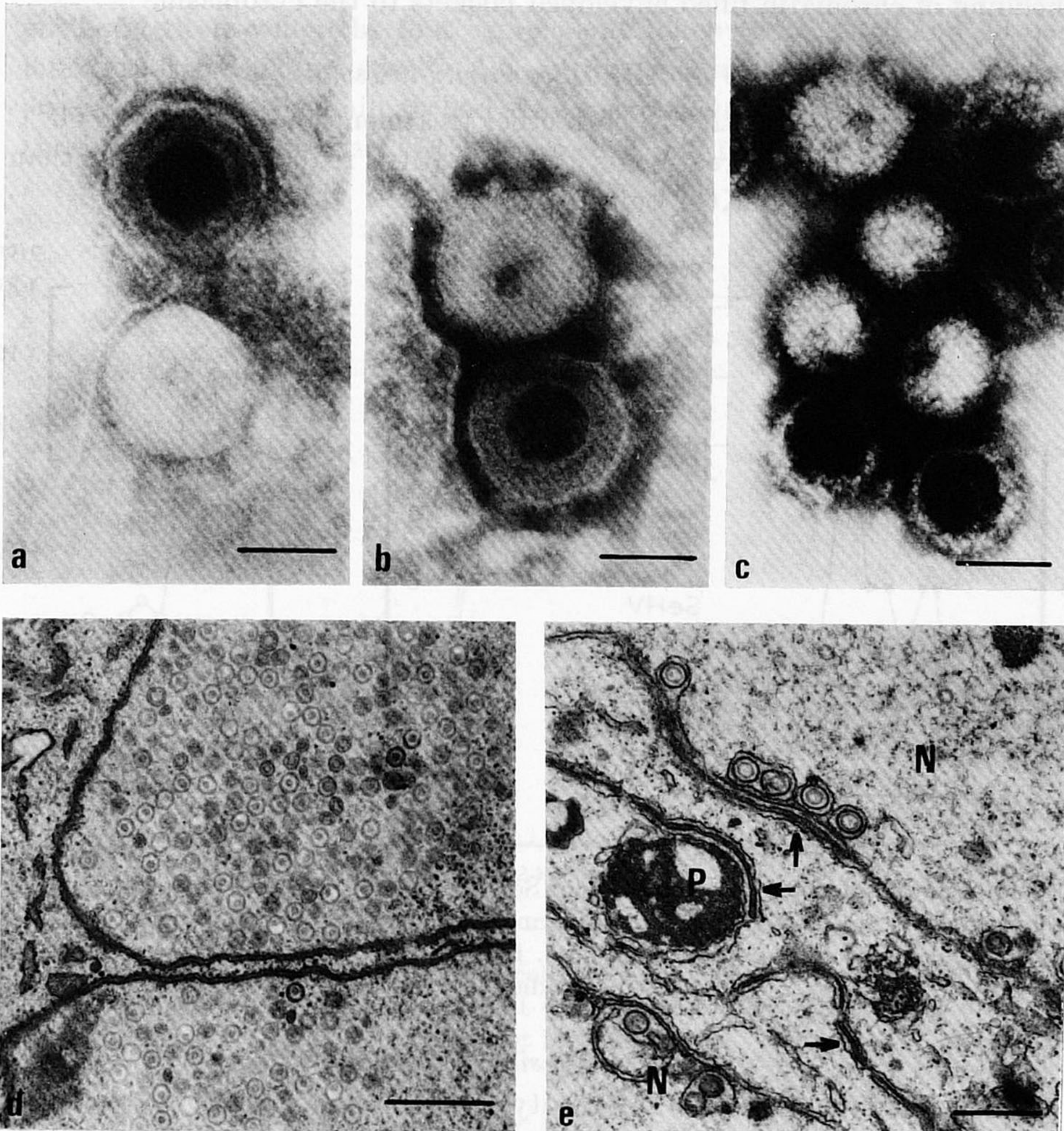


Fig. 2. In *a*, *b* and *c* enveloped and naked herpes virus particles are shown as revealed by negative contrast electron microscopy. Bar markers represent 100 nm. *d* and *e*: thin-section transmission electron micrographs demonstrating the presence of viral capsids in nuclei of infected cultured kidney cells. Bar markers represent 500 nm. In *d*, naked capsids mostly showing a core are seen lying within the matrix of two closely apposed nuclear lobes. In *e*, enveloped viral capsids are shown lying in the perinuclear space of two nuclear sections (*N*) in one cell. Note reduplication of nuclear envelope membranes (arrow) and the presence of a nuclear pocket containing condensed chromatin (*P*) in the cytoplasm

The nuclear changes in particular were very notable. Briefly, these included: margination and disappearance of heterochromatin, prominent reduplication of nuclear envelope, presence of abundant intranuclear membranous lamellae, occurrence of various kinds of nuclear inclusions, such as tubular structures, vermicellar bodies and bundles of filaments, and formation of nuclear pockets including condensed chromatin with shedding of chromatin-filled enveloped pockets to the cytoplasm.

Ether and Heat Sensitivity to Isolate

The isolate was sensitive to both ether treatment and exposure to 56° C for 30 minutes. Both treatments caused a titre reduction of more than 4.0 log₁₀ TCID₅₀ for SeKC-2 cultures.

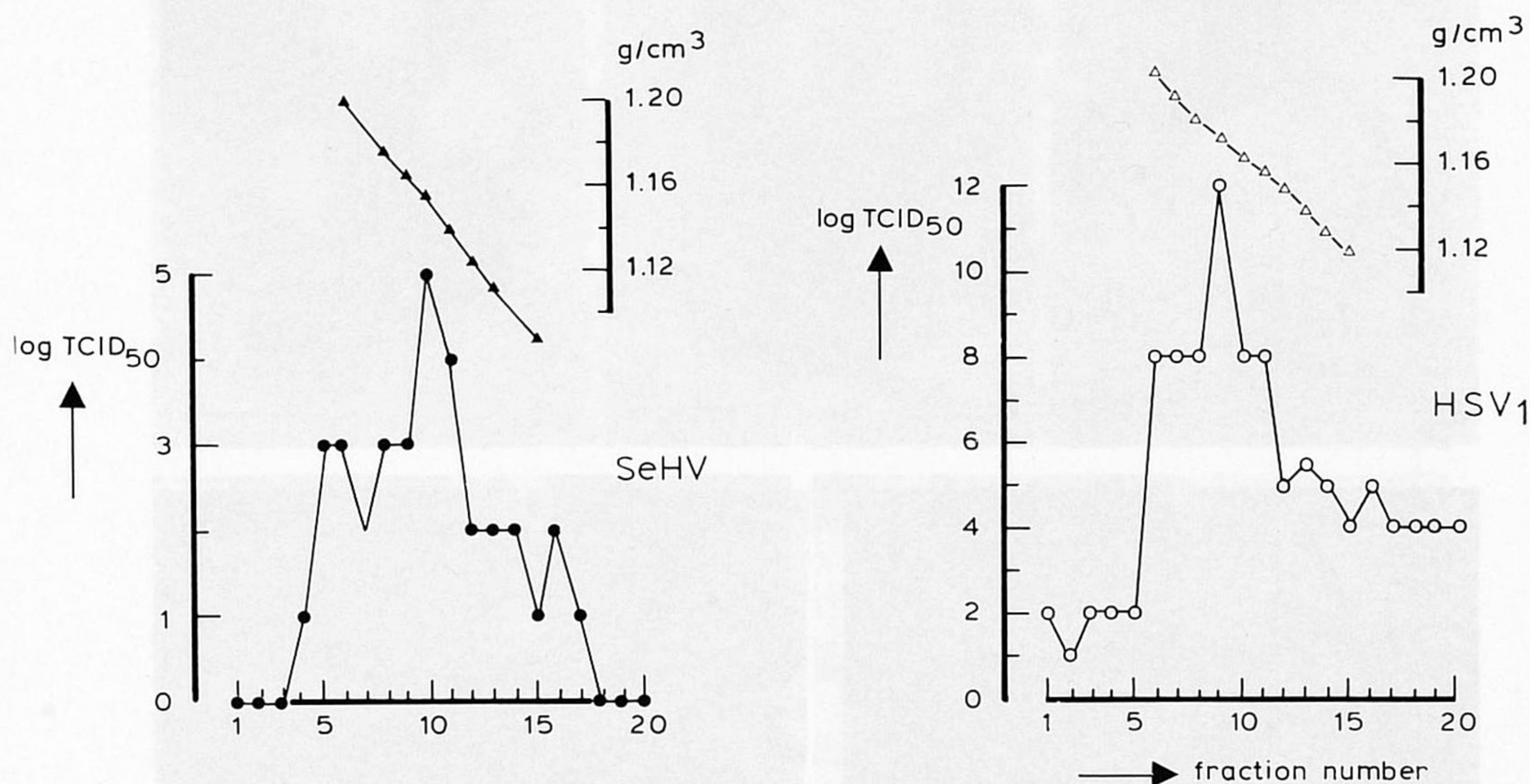


Fig. 3. Buoyant density determination of SeHV in a sucrose gradient at 20° C. Virus concentrations in gradient fractions determined in the infectivity test and buoyant density calculated from refractive indices. HSV-1 served as a reference in a parallel gradient

Buoyant Density in Sucrose

After fractionation and infectivity titration the density of the peak fractions of both sucrose gradients used for the comparison of the buoyant densities of SeHV and HSV-1, were calculated from their respective refractive indices. The buoyant densities of both SeHV and HSV-1 proved to be in the same order of magnitude: 1.16 and 1.17 g/ml respectively (Fig. 3).

Replication Cycle in SeKC-2 Culture

Both in culture media and in cell lysates new virus was first detected 16 hours after infection (Fig. 4). Thereafter the concentrations in both

fractions rose rapidly. Cell free virus concentrations reached a plateau at about 36 hours after infection and cell-associated virus concentrations at about 50 hours after infection.

Serology with Paired Serum Samples from Seals

The paired serum samples collected from 10 seals were tested in the VN test on SeKC-2 cultures with SeHV. Seven animals showed no detectable antibody titers when the first sample was taken (titre <10) and 20 days later in all except one specific VN antibody (titre ≥ 10) could be demonstrated. Seven of the animals had shown seroconversion after 20 days (more than twofold rise in titer) in the VN test (Table 1).

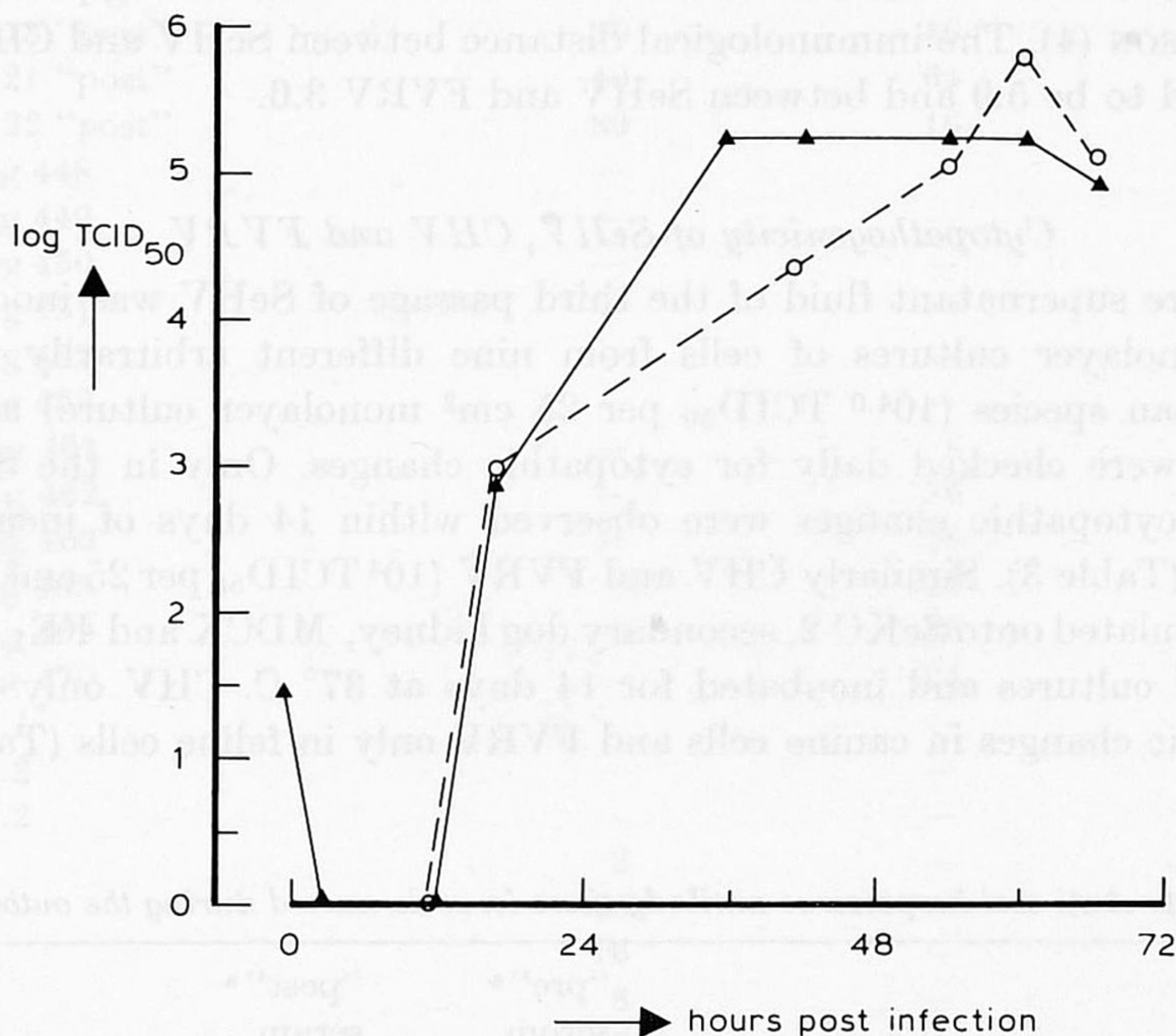


Fig. 4. SeHV growth cycle in SeKC-2 cells. Cell associated (\blacktriangle — \blacktriangle) and culture fluid (\circ — \circ) virus concentrations at different times after infection determined in the infectivity test in SeKC-2 cells

Antigenic Relationship with Other Members of the Herpesviridae Family

Antiserum preparations against a selected number of the established members of the Herpesviridae (see Materials and Methods) were tested in the VN test on SeKC-2 cultures using 50—100 TCID₅₀ SeHV. Apart from antisera directed against canine herpesvirus (CHV) and feline herpesvirus (FVRV) no VN antibody titers were found in the panel of reference sera. Results of cross-neutralization tests between SeHV, CHV and FVRV, carried out in the respective VN tests are shown in Table 2. All the nine

seal sera which neutralized SeHV, also neutralized CHV, although no clear correlation in titers was found. Only six out of these nine sera also neutralized FVRV. Only two out of six canine sera which neutralized CHV neutralized SeHV although with very low titers. None of the dog sera neutralized FVRV. All five cat sera which neutralized FVRV also neutralized SeHV although the heterologous titers tended to be lower. None of the cat sera neutralized CHV. None of the sera from these three species, which were negative in the homologous VN test were positive in any of the heterologous tests. Thus, although no antigenic relationship could be demonstrated in the VN tests with these sera between CHV and FVRV, both viruses proved antigenically related to SeHV. On the basis of these results, immunological distances between the two viruses and SeHV were calculated according to HONNESS and WATSON (4). The immunological distance between SeHV and CHV was calculated to be 5.9 and between SeHV and FVRV 3.6.

Cytopathogenicity of SeHV, CHV and FVRV

Culture supernatant fluid of the third passage of SeHV was inoculated onto monolayer cultures of cells from nine different arbitrarily chosen mammalian species ($10^{4.0}$ TCID₅₀ per 25 cm² monolayer culture) and the cultures were checked daily for cytopathic changes. Only in the SeKC-2 cultures cytopathic changes were observed within 14 days of incubation at 37° C (Table 3). Similarly CHV and FVRV (10^4 TCID₅₀ per 25 cm² bottle) were inoculated onto SeKC-2, secondary dog kidney, MDCK and FE₁ monolayer cell cultures and incubated for 14 days at 37° C. CHV only caused cytopathic changes in canine cells and FVRV only in feline cells (Table 3).

Table 1. *Anti-seal herpesvirus antibody titers in seals nursed during the outbreak*

Seal no.	"pre" ^a serum titre	"post" ^a serum titre
84-03	10	80
84-04	<10	10
84-06	<10	<10
84-09	<10	10
84-11	<10	40
84-12	<10	160
84-18	10	10
84-21	20	40
84-22	<20	80
84-23	<20	160

^a "pre" serum samples collected seven days after the first seal had died and "post" serum samples collected 20 days later

Table 2. *Micro-VN titers in sera from SPF dogs, cats and seals against SeHV, CHV and FVRV^a*

Antiserum	Virus		
	SeHV	CHV	FVRV
seal 84-22 "pre"	—	—	—
seal 84-23 "pre"	—	—	—
seal 84-03 "post"	80	32	16
seal 84-04 "post"	10	16	2
seal 84-09 "post"	10	8	4
seal 84-11 "post"	40	4	2
seal 84-12 "post"	160	64	32
seal 84-15 "post"	80	8	8
seal 84-18 "post"	10	16	—
seal 84-21 "post"	40	64	—
seal 84-22 "post"	80	16	—
SPF dog 448	—	—	—
SPF dog 449	—	—	—
SPF dog 450	—	—	—
SPF dog 451	—	—	—
SPF dog 452	—	—	—
SPF dog 453	—	—	—
SPF dog 461	—	8	—
SPF dog 462	—	16	—
SPF dog 463	—	16	—
SPF dog 465	4	32	—
SPF dog 469	4	16	—
SPF dog 470	—	64	—
cat 30.1	—	—	—
cat 31.2	—	—	—
cat 32.2	—	—	—
cat 59	8	—	32
cat 60	16	—	16
cat 102	16	—	32
cat 169	8	—	16
cat 273	8	—	32

^a — = titer < 2Table 3. *Presence (+) or absence (—) of cytopathic changes after inoculation of SeHV, CHV and FVRV in seal, canine and feline cells*

Viruses	Cell cultures			
	SeKC-2	MDCK	Secondary dog kidney	Fe ₁
SeHV	⊕	—	—	—
CHV	—	⊕	⊕	—
FVRV	—	—	—	⊕

Discussion

The aetiological role of SeHV for the acute disease with high mortality in the seals was suggested by the isolation of SeHV from organ suspensions of most animals which had died with the same symptoms and exhibiting the same pathological changes. Moreover the virus could not be isolated from the seal which had obviously died from another cause outside the orphanage. Most of the seals in the orphanage showed clear seroconversion to SeHV during the outbreak as demonstrated in the VN test (Table 1). The clinical manifestations and pathology of the disease were dominated by an acute pneumonia and a focal hepatitis, although also acute gingivostomatitis was observed in some of the animals. These are all features not uncommon in generalised infections with other Alphaherpesvirinae members especially in young mammals (1). The pathogenesis and pathology of the disease are subject of further study and will be reported elsewhere. It is interesting to note that the absence of influenza A (H₇N₇) virus infection — another agent causing severe pneumonia and high mortality in seals (3) — was serologically demonstrated during these investigations (data not shown).

SeHV was tentatively classified as a member of the Herpesviridae family on the basis of its characteristic morphology in electron microscopy, its buoyant density in sucrose and its sensitivity to ether and heat treatment. The apparent antigenic relationship with CHV and FVRV (immunological distances of 5.7 and 3.6 respectively), its relatively short replication cycle and the cytopathic changes caused in SeKC-1 and SeKC-2 cultures, suggested that SeHV should be considered a probable member of the Alphaherpesvirinae subfamily. The serologic data and the restricted cytopathogenicity indicated that SeHV is probably not one of the herpesviruses previously recognised in other animal species. Although antigenically related, SeHV is distinct from both CHV and FVRV. With CHV it seems to share an almost "one way" antigenic relationship: only two out of six canine anti-CHV sera neutralized SeHV to a limited extent, whereas all the nine seal anti-SeHV sera neutralized CHV. In contrast all five feline anti-FVRV sera neutralized SeHV, but only six out of nine seal anti-SeHV sera neutralized FVRV. Despite the absence of an antigenic relationship between CHV and FVRV on the one hand, both these viruses are antigenically related to SeHV on the other hand. This indicates that probably different viral membrane glycoproteins or at least different epitopes are involved in the cross-neutralization assays with SeHV. These findings may have consequences for the evaluation of the phylogenetic relationship between these three viruses. Further studies are needed to characterize SeHV on the basis of its biological properties and genome structure. Also the identification of any polypeptides showing conservation of sequences with analogous products in cells infected with other viruses of the subfamily should be attempted by

immune precipitation studies with the aim to come to the final hierarchical classification of SeHV.

In conclusion, the present data indicate that the herpesvirus isolated from the harbor seal named seal herpesvirus (SeHV) or phocid herpesvirus 1 — according to the recommendations of the Herpesvirus study group of the International Committee on Taxonomy of Viruses (7) — is a new member of the Herpesviridae family.

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