Acta virol. **26** : 256—263, 1982

GROWTH CHARACTERISTICS OF HERPESVIRUSES ISOLATED FROM FREE LIVING SMALL RODENTS

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Received December 5, 1981

Summary. — Sixteen cell cultures of different origin (bird, rodents, carnivores, pigs, monkey, man) reproduced five strains of herpesviruses isolated from small free living rodents *Apodemus flavicollis* and *Clethrionomys glareolus* revealing cytopathic changes typical for members of the family *Herpesviridae*. The virus titres in different cell cultures ranged from 10^2 to 10^7 TCD_{50} per ml. The growth curves of two isolates originating from both animal species resembled to those obtained with human herpesviruses type 1 and 2, pseudorabies virus and guinea pig herpes-like virus (Hsiung-Kaplow) in identical cell cultures. Mouse cytomegalovirus was grown practically in mouse embryo fibroblasts only. The five isolates from free living small rodents were classified according to cytopathic changes and growth characteristics in different cell cultures as members of the subfamily *Alphaherpesvirinae*.

Key words: *Alphaherpesvirinae*; *free living rodents*; *growth characteristics in cell cultures*

Introduction

Previous reports (Blaškovič *et al.*, 1980, Čiampor *et al.*, 1981) on five virus isolates from free living small rodents confirmed that the isolated strains belong to the family *Herpesviridae*. However, no decision was made, whether they are members of the subfamily *Alphaherpesvirinae* or *Betaherpesvirinae*. Considering that the cytomegaloviruses (subfamily Betaherpesvirinae) prefer mainly species specific homologous cell cultures for their growth in vitro (Diosi and Babusceac, 1970, Diosi *et al.*, 1972, Kim *et al.*, 1975), we attempted to prove the growth requirements and characteristics of three strains of herpesviruses isolated from *Clethrionomys glareolus* and of two strains from *Apodemus flavicollis*.

Materials and Methods

Cell cultures and media. Primary mouse embryo cells (MEC) from 16—18 days old embryos, rabbit kidney cells (RKC) from an adult animal and chick embryo cells (CEC) were obtained by homogenization and trypsinization of the respective tissues. Continuous cell cultures with a history

of many successive passages were supplied from the collection of the Institute of Virology, Bratislava. The origin of the most common cell lines is not mentioned.

Following cell cultures were used: AM_{57} — epithelial cells derived from human amnion (Mayer and Mayerová, 1958), BHK-21 (baby hamster kidney cells), CCL-64 — epithelial cells from mink lung tissue (Henderson *et al.*, 1974), GMK — epithelial cells from the kidney of *Cercopithecus aethiops* monkey, HeLa — epithelial cells of human cervical carcinoma, L — fibroblast like cells from mouse connective tissue, LEP — fibroblast like cells from the lung of human embryo (kindly supplied by D. Řezáčová, Institute of Sera and Vaccines, Prague), GPT — fibroblast like cells from guinea pig tongue (Svobodová *et al.*, 1977), PS — epithelial cells from pig kidney (Korych, 1960), REF — rabbit embryo fibroblasts (kindly supplied by D. Řezáčová, Institute of Sera and Vaccines, Prague), SIRC — epithelial cells from rabbit cornea, VERO-fibroblast like cells from the kidney of Cercopithecus aethiops monkey, Zp — fibroblast like cells from rabbit lung (Szántó, 1960). The cells were grown in basal Eagle medium (BEM) with 5—10% heat-inactivated calf of bovine serum (ICS or IBS respectively). Earle's medium (EM) with 5% ICS was used for primary cell cultures. 100 units per ml penicillin and 100 μg/ml streptomycin were added to each medium.

Viruses. The three strains of herpesviruses isolated from *Clethrionomys glareolus* (Nos. 60, 68, 72) and the two strains isolated from *Apodemus flavicollis* (Nos. 76 and 78) revealed a titre of approximately 10^7 TCD_{50} per ml in REF (Blaškovič *et al.*, 1980). Human herpesvirus type 1 (HSV 1) and type 2 (HSV 2) passaged on ZP cell cultures (20th and 47th respectively) were obtained from the collection of the Institute of Virology, Bratislava. Guinea pig herpes like virus (GPHLV) was kindly supplied by Professor G. D. Hsiung, University of HNew Haven, U.S.A. (Bia *et al.*, 1980). Infectious bovine rhinotracheitis virus (IBR), strain R-6 (Menšík and Rozkošný, 1969) originated from the Research Institute for Veterinary Medicine, Brno. Mouse salivary gland virus (MSGV — mouse cytomegalovirus), strain Smith (1954) was obtained from American Type Culture Collection, Rockville, Md., U.S.A., by courtesy of WHO. Pseudorabies virus (PRV), attenuated strain CK — PRV X (Svobodová, 1968) and PRV virulent strain TOP (Golais and Sabó, 1975) were supplied by the Institute of Virology, Bratislava.

Virus titration.;Virus titration was performed in test tubes. The cytopathic effect (CPE) was checked daily from 2 to 12 days post infection (p. i.). End point was calculated according to Reed-Munch and expressed in TCD_{50} per 1 ml or per 0.1 ml, respectively. Cell cultures were inoculated with 0.1—0.2 ml viral suspension per tube. REF cultures employed (were infected at a multiplicity of 1 TCD_{50} per cell. The stock virus was obtained after three times freezing and thawing of infected cells and kept at —70° C.

Preparation of cell cultures for cytological examinations. Infected cell cultures as well as uninfected controls grown on glass cover slips were removed from the tubes at different time intervals, fixed in Carnoy solution for 15—60 min and then transferred to 96% alcohol for one or more days. The fixed specimens were stained with hematoxylin-eosine.

Results

Interaction of herpesvirus isolates with cell cultures of different origin

Sixteen different cell cultures of animal and human origin were infected with the isolates Nos. 60, 68, 72, 76 and 78. The susceptibility of cell cultures to the isolates was different. At the beginning, CEC, L, ZP, BHK-21, HeLa and LEP cell cultures revealed low susceptibility to viruses in question so that their titres could be read after blind passages only. Table 1 shows that after a few successive passages the L and GPT cell cultures became sufficiently susceptible yielding virus titres $10^{5.5}$ TCD_{50}/ml; in BHK-21 cells a titre of 10^7 TCD_{50}/ml was reached. These as well as REF cells were used for virus propagation and for serological and biochemical procedures. Infectious titres reached in both cultures were approximately identical for each of the isolates used.

Table 1. Growth of the isolates in different cell cultures

Type of cell culture designation	origin	No. of passages blind	for titration	Max. inf. titre* TCD_{50}/ml	Type of CPE (its appearance in days)
CEC**	chick	3	7	2	CR (3)
L	mouse	2	30	5.5	CR (2.2)
MEF**	mouse	0	1	3.5	
RKC**	rabbit	0	3	3	CR (4.5)
SIRC	rabbit	0	3	3	CR (3.75)
ZP	rabbit	2	30	3	CR
REF	rabbit	0	50	7	CR (2.5)
BHK-21	hamster	1	15	6	CR (4.5)
GPT	guinea pig	0	20	5.5	PKC (5.1)
CCL-64	mink	0	3	2	CR (3)
PS	swine	0	5	4	CR (5)
GMK	monkey	0	5	3	CR (2.5)
VERO	monkey	0	12	4.5	CR (3.5)
Am-57	human	0	3	3	CR (4)
HeLa	human	2	5	2	CR (4)
LEP	human	2	5	2	CR (3.5)

a* Infectious titres indicate the $\log_{10}$ value reached in the last passage.
** primary cell culture.
CR — cell rounding; PKC — polykaryocytes.

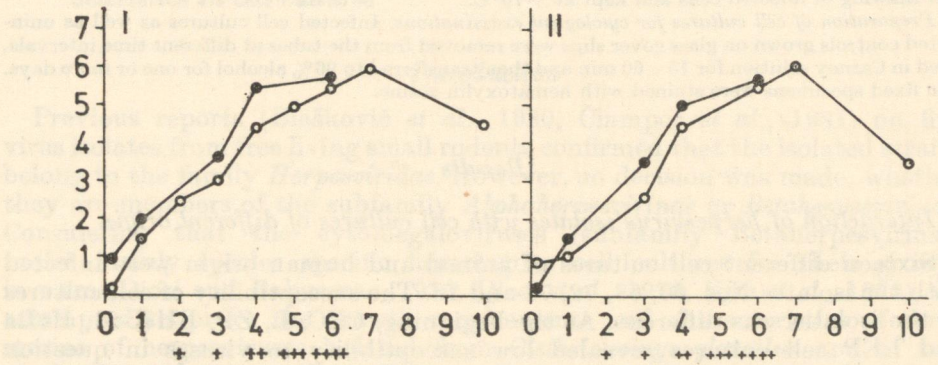

Fig. 1.

Growth curves of the herpesvirus isolates No. 72 (I) and No.(76 (II) in REF cell culture
Abscissa: days p. i. and the degree of CPE (+, ++, +++, ++++)
Ordinate: virus titre in log TCD_{50}/0.1 ml
cell-free virus ○ — — ○
cell-associated virus ● — — ●

Growth characteristics of the isolates in REF cell culture

Growth characteristics of two isolates of different origin, namely No. 72 from *Clethrionomys glareolus* and No. 76 from *Apodemus flavicollis*, were followed for different time intervals in REF cell cultures. The intracellular virus (ICV) titre always surpassed the value of the extracellular virus (ECV) in the course of six days p. i. The rise in both ICV and ECV correlated directly with the CPE formation as observed in light microscope by 48 hrs p. i. At this

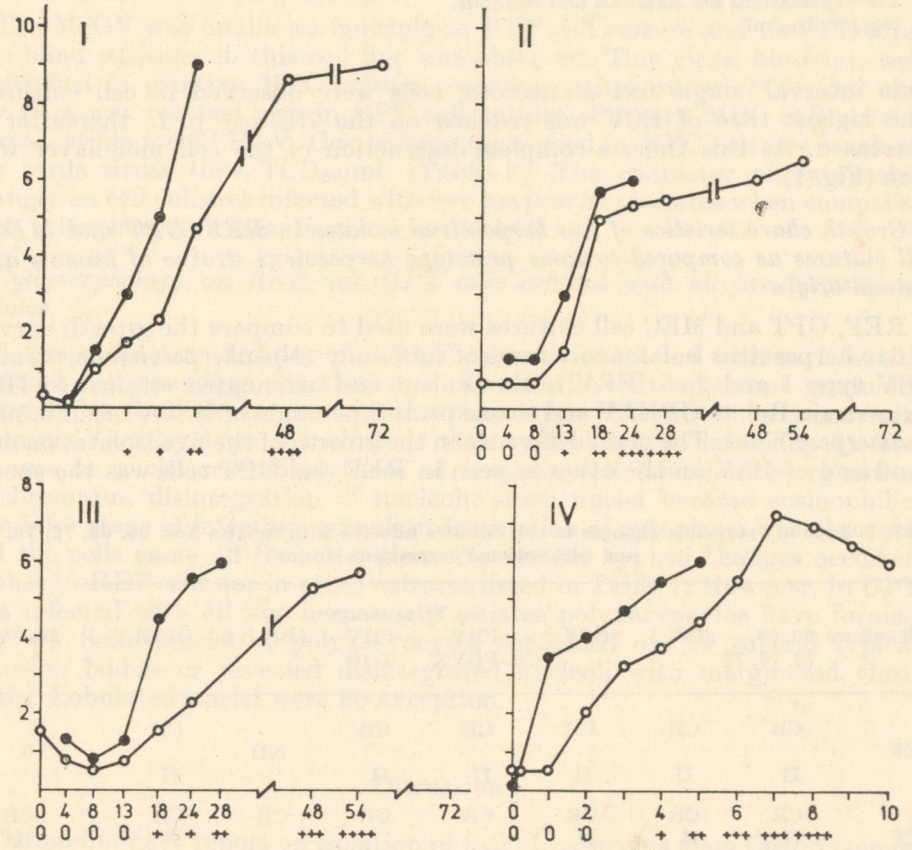

Fig. 2.

Growth curves of the prototype viruses in GPT cell culture

I — HSV type 1
II — PRV virulent strain TOP
III — PRV attenuated strain CK-PRVX
IV — GPHLV
For further explanations see Fig. 1

Table 2. Infectious titres of the five virus isolates and prototype strains HSV type 1 and virulent PRV

Cell lines	Virus strains*								
	60	68	72	76	78	HSV 1	TOP	GPHLV	MSGV
REF	6**	5.5	6.5	6.5	7	6.5	5.5	4.5	0
GPT	3.5	2.5	3.5	3.5	3.5	3.5	7.5	6.5	1.7
MEC	ND	ND	3.5	3.5	ND	ND	ND	ND	6

ND — not done.
* For explanations see Materials and Methods.
** $\log_{10}$ TCD_{50}/ml.

time interval, single foci of necrotic cells were observed in cell cultures, The highest titre of ECV was reached on the 7th day p. i., thereafter it decreased. At this time, a complete destruction of the cell monolayer was seen (Fig. 1).

Growth characteristics of five herpesvirus isolates in REF, GPT and MEC cell cultures as compared to some prototype herpesvirus strains of human and animal origin

REF, GPT and MEC cell cultures were used to compare the growth curves of five herpesvirus isolates to viruses of subfamily *Alphaherpesvirinae*, strains HSV type 1 and 2, to PRV both virulent and attenuated strains, to IBR virus strain R 6, to GPHLV and to one prototype strain of MSGV of subfamily *Betaherpesvirinae*. The main difference in the growth of the five isolates on one hand and of HSV on the other as seen in REF and GPT cells was the speed

Table 3. Type of cytopathic changes in cell cultures infected with isolates Nos. 60, 68, 72, 76, 78 and with selected herpesvirus strains

Cell culture	Virus strain							
	60, 68, 72, 76, 78	HSV 1	HSV 2	PRV CK-PRVX	PRV TOP	IBR R 6	GPHLV	MSGV
REF	CR II	CR II	CR II	CR II	CR II	ND	CR II	0
GPT	CR II PKC	CR II PKC	CR II PKC	CR	CR PKC	CR PKC	CR II	CR II
MEC	CR II	ND	ND	ND	ND	ND	0	CR II

CR — cell rounding; II — intranuclear inclusions; PKC — polykaryocytes; 0 — no growth; ND — not done

of virus multiplication and CPE formation. The CPE of HSV 1 and HSV 2 appeared between 8—13 hr and within 18—48 hr it became complete. The highest titre of these viruses expressed in TCD_{50}/0.1 ml was reached up to 24 hr p. i. The CPE of five isolates developed slower and has not reached comparable high titres. Growth curve of HSV 1 in GPT cell culture is presented in Fig. 2/I. The comparison of virus multiplication of the virulent an attenuated strains of PRV is presented in Figs. 2/II and 2/III. The growth curve of GPHLV on GPT cell cultures resembles that of the isolates Nos. 72 and 76 on REF cells (Fig. 2/IV).

The MSGV was unable to multiply in REF cell culture and no CPE after two blind passages in this cell line was observed. This virus, however, well multiplied in primary MEC culture reaching a titre of 10^6 TCD_{50}/ml and low titre was reached also in GPT cell culture. Primary MEC culture was unsusceptible for GPHLV. Our isolates multiplied in MEC cultures reaching low virus titres $10^{3.5}$ TCD_{50}/ml. (Table 2). The character of cytopathic changes on cell cultures infected with five herpesvirus isolates when compared with other herpesviruses, is given in Table 3.

Cytomorphology on REF and GPT cells infected with all five herpesvirus isolates.

Between the 1st - 3rd day p. i. REF cultures inoculated with each isolate revealed cell rounding. Further foci of necrotic cells appeared gradually followed by total destruction of the monolayer. The nuclei of some cells contained Cowdry type A inclusions with typical bright halo (Fig. 3). The changes in nuclei resembled those of herpetic cell infection: margination of chromatin, disintegration of nucleoli; some nuclei became eosinophilic. At a later stage of infection, complete destruction of cytoplasm was observed and the cells came off from the glass. No further typical changes occurred neither in REF cells nor in other cultures listed in Table 1. However, in GPT cells infected with all five herpesvirus isolates polykaryocytes have formed (Fig. 4). Some nuclei of polykaryocytes contained one or several typical inclusion bodies or revealed disintegrated nucleoli with marginated chromatin. Lobulated nuclei were no exception.

Discussion

The preliminary report on isolation of five virus strains from *Clethrionomys glareolus* (Nos. 60, 68 and 72) and *Apodemus flavicollis* (Nos. 76 and 78) small free living rodents suggested that the viruses isolated belong to the family *Herpesviridae* (Blaškovič *et al.*, 1980). Electronmicroscopic studies (Čiampor *et al.*, 1981) definitely characterized the virions produced in REF cultures as herpesviruses. Despite the fact, that the ultrastructural development of the virus isolates in cells resembled that of freshly isolated HSV type 1, the tubular structures in nuclei of infected cells resembled to those seen by cytomegaloviruses (Čiampor *et al.*, 1981).

The growth characteristics of herpesvirus isolates on cell cultures of different origin (human, animal) should have a decisive role in differentiation whether they belong to the subfamily *Alpha-*, or *Betaherpesvirinae*. It was generally believed that cytomegaloviruses multiply in their species specific cell cultures (Plummer, 1967). This assumption became doubtful as the growth of human cytomegalovirus on Vero cells (Demidova *et al.*, 1972) or the CMV from *Cercopithepus aethiops* in human cell cultures (Dejčman *et al.*, 1972) was claimed to be successful.

Kim and Carp (1971) found that murine cytomegalovirus was grown in green monkey kidney cells (BSC-1), primary RKC, BHK 21 cells, L cells and in primary fetal sheep brain cells. In some cases the CPE was produced after several successive passages only. However, no growth in five cell lines of human origin was observed. Previous finding of Raynaud *et al.*, (1969, 1972) that the murine cytomegalovirus was grown in cells of human origin, was not confirmed by Kim *et al.* (1974). Human diploid cells were able to produce with murine cytomegaloviruses an abortive infection only (Kim and Carp, 1972).

In our experiments, using 16 different cell cultures of human and animal origin (Table 1) arranged according to the position of the host species in the evolutionary ladder (birds, rodents, carnivora, pigs, monkeys, human) it was found that all cell cultures were susceptible for the multiplication of isolates. Those cell cultures, derived from rodents, were found according to the virus titres achieved, to be the most suitable. On the other hand, we admit, that the quantitative estimation of virus titre could depend upon the number of subpassages performed on respective cell culture. Nevertheless, wee consider this preliminary information as interesting one.

Regarding to the susceptibility of cells from different animal species to the isolates regarding to the results of electronmicroscopic studies (Čiampor *et al.*, 1981), the course of growth curves in REF, MEC and GPT cell cultures and the appearance of CPE in these cell cultures, we feel to be authorized to classify our herpesvirus isolates as the members of subfamily *Alphaherpesvirinae*. We were unable to compare our isolates with alphaherpesviruses isolated from small free living rodents, because all viruses isolated and described from these animal species so far, were classified as cytomegaloviruses (Diosi and Babusceac, 1970, Diosi *et al.*, 1972, Smith, 1954, Raynaud *et al.*, 1972). These cytomegaloviruses isolated from small rodents, referred to Diosi and Raynaud strains, were compared with the original and prototype (Smith, 1954) strain of MCMV, and were foudn identical according to their biological and biochemical properties (Kim *et al.*, 1975).

Acknowledgement: We are grateful to Mrs. E. Trenčianská for skilful technical assistance.

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Explanation of Micrographs (Plates XXXII–XXXIII)

Fig. 3. Cytopathic changes in REF cell culture infected with the herpesvirus isolate No. 60. × 500.
Fig. 4. Polykaryocytes in GPT cell culture infected with the herpesvirus isolate No. 60. × 500.