

NEW MODEL CELL SYSTEMS (PK AND XTC-2) FOR STUDYING ACUTE AND PERSISTENT INFECTIONS WITH HERPES SIMPLEX AND PSEUDORABIES VIRUSES

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Summary. — Herpes simplex virus type 1 (HSV-1) showed limited replication in PK (pig kidney) and XTC-2 (*Xenopus laevis* frog) cell lines. Virus replication depended on the multiplicity of infection (MOI). At a high MOI, HSV-1 caused a typical cytopathic effect (CPE) in XTC-2 cells but a little marked CPE in PK cells. Pseudorabies virus (PRV) replicated intensively in PK cells (permissive system) but not in XTC-2 cells (nonpermissive system). Both viruses were adsorbed on to PK and XTC-2 cells. In infected PK cells, fluorescent HSV-1 antigen was demonstrated only in the vicinity of the nuclear membrane and in the paranuclear area of the cytoplasm but not in the nuclei. In XTC-2 cells, HSV-1 antigen was demonstrated also in the nuclei. Persistent HSV-1 infection was induced in PK but not in XTC-2 cells; it was of limited duration. PK cells which had lost HSV-1 multiplied further and proved susceptible to infection with HSV-1 or PRV.

Key words: herpes simplex virus; pseudorabies virus; restrictive and nonpermissive infection; persistent infection; immunofluorescence

Introduction

New data on the interaction of herpesviruses with their hosts on the cellular and molecular levels have been accumulating rapidly. These interactions are usually characterized by the fact that acute infection of the organism is followed by a latent or persistent infection frequently accompanied by recurrent outbreaks of the infection. Research on herpesviruses is thus of particular importance not only for various biological disciplines including virology, molecular biology, genetics and evolution but also for clinical and veterinary medicine. The herpesvirus group includes members differing in their antigenic relatedness which needs not be related to the range of their experimental host animals or the range of susceptible cells (Wildy, 1973; Darlington and Granoff, 1973).

Table 1. ICV titres of HSV-1 strains in PK and XTC-2 cells in comparison with their titres in ZP cells

Cell line	HSV-1 strain	ICV titres (log TCID ₅₀ /ml) after inoculation at the indicated MOI (TCID ₅₀ /cell)				
		3	0.3	0.03	0.003	0.0003
PK	HSZP	2.33	1.0	0	0	0
	MA	3.66	1.23	0	0	0
XTC-2	HSZP	2.33	1.33	<1.0	0	0
	MA	4.66	4.0	3.33	2.0	0
ZP	HSZP	7.0	8.33	7.0	6.66	6.66
	MA	6.66	8.0	7.5	7.33	7.33

0 means no infectious ICV demonstrated.

The titres were determined 48 hr p.i.

We investigated the interaction of HSV-1 and PRV with little susceptible cells from the point of view of 1) the cell substrate, 2) the synthesis of infectious virus and fluorescent antigen, and 3) acute infection and the possibility of inducing a persistent infection. The data obtained were compared with findings in a permissive cell system.

Materials and Methods

Viruses. HSV-1 strains HSZP and MA passaged in the ZP (rabbit lung) cell line (Szántó *et al.*, 1972) and PRV strains TOP, DK-M (both virulent) and TK-900 (vaccine) passaged in chick embryo cells (CEC) (Golais and Sabó, 1975) were used.

Cell cultures. The PK pig kidney cells (Korych, 1960) were grown at 36 °C in synthetic medium supplemented with two protein fractions of calf serum (0.375%) albumin and 0.025% specific alpha-globulin) and lactalbumin hydrolysate (Michl, 1962) to which 5% inactivated calf serum (ICS) was added. XTC-2 cells, a continuous cell line derived from the frog *Xenopus laevis* (Pudney *et al.*, 1973), were grown at 28–30 °C (if not stated otherwise) in Eagle's (1955) basal medium supplemented with 5% ICS. For passaging, the cells were dispersed with a mixture of equal volumes of trypsin (0.25%) and sodium EDTA (0.02%) solutions.

Infectious virus assay. In PK and XTC-2 cell cultures, both intracellular (cell-associated) virus (ICV) and virus released into the medium (ECV) were assayed. HSV-1 and PRV were titrated in tube cultures of ZP cells and CEC, respectively (titres expressed in TCID₅₀/ml values) or by the plaque assay in Vero cells under methyl-cellulose overlay (titres expressed in PFU/ml values).

Immunofluorescence. The indirect technique was used. For the detection of HSV-1 antigens rabbit immune sera against the nucleocapsid, viral envelope or complete virus and pig anti-rabbit (SwAR) fluorescein isothiocyanate- (FITC-) labelled conjugate were employed. PRV antigens were detected with immune pig serum and FITC-labelled rabbit anti-pig conjugate (RASw). The preparation of immune sera against HSV-1 components was described (Leššo *et al.*, 1976). Micrographs were taken on ORWO RS 2 film in a Fluoval (C. Zeiss, Jena) microscope.

Results

Replication of HSV-1 and PRV in PK and XTC-2 cells

Replication of HSV-1 in PK and XTC-2 cells was restricted as compared with that in the fully susceptible ZP cells. It was markedly dependent on the

Table 2. Synthesis of ICV and ECV in HSV-1-infected PK cells grown in medium with and without ICS

HSV-1 strain	log TCID ₅₀ /0.1 ml in inoculum	MOI (TCID ₅₀ /cell)	Titres (log TCID ₅₀ /ml) in			
			medium + ICS		medium - ICS	
			ECV	ICV	ECV	ICV
HSZP	7.0	15	4.0	4.5	5.33	5.33
	6.5	5	3.66	2.66	4.66	3.33
MA	7.0	15	3.66	4.66	5.5	5.5
	6.0	4	2.33	2.0	3.33	3.0

MOI, in PK cells more than in XTC-2 cells. At low MOI no infectious HSV-1 was synthesised (Table 1). At remote intervals the virus titres remained unchanged or were gradually decreasing. In ZP cells, high MOI (undiluted virus) resulted in the formation of defective interfering particles (DIP); the titre of infectious virus namely was lower than after inoculation with diluted virus.

The differences between ICV and ECV titres in PK cells varied considerably (Table 2). The results also showed that the yield of infectious HSV-1 was in most cases from 10- to 100-fold higher in PK cells maintained in medium without ICS. However, in some experiments the difference was minimal.

PK cells proved to be fully susceptible to infection with both virulent and attenuated PRV (Golais and Sabó, 1976), while XTC-2 cells were fully resistant (Table 3). In one experiment a low level of infectious virus was found in XTC-2 cells infected with the attenuated (vaccine) strain TK-900 of PRV.

Immunofluorescence detection of HSV-1 and PRV antigens in PK and XTC-2 cells

In PK cells infected with strains HSZP and MA HSV-1 antigen was demonstrated in the form of brightly fluorescent granules in the vicinity of the nuclear membrane and as diffuse fluorescence in the paranuclear

Table 3. ECV titres in PK and XTC-2 cells infected with virulent and attenuated PRV strains

Cell line	PRV strain	ECV titres (log TCID ₅₀ /ml) after inoculation with the indicated MOI (TCID ₅₀ /cell)			
		5	0.1	0.1	0.001
PK	DK-M	n.d.	8.0	8.0	6.5
	TK-900	n.d.	8.33	7.66	6.5
XTC-2	TOP	0	0	n.d.	n.d.
	TK-900	3.33	0	n.d.	n.d.

0 = no infectious ECV demonstrated.

n.d. = not done.

Table 4. Immunofluorescence detection of viral antigens in cells

Cell line	MOI TCID ₅₀ /cell	Hr p.i.	HSV-1 HSZP		HSV-1 MA		PRV TOP, TK-900
PK	10	24	+		++		+++
		48	++		+++		+++
		72	+		+		n.d.
			30 °C	36 °C	30 °C	36 °C	30 °C
XTC-2	2	16	+	++	+	++	0
		24	++	+++	++	+++	0
		48	++	+++	++	+++	0

0, +, ++ and +++: no, 5–10 %, 20–60 % and 70–100 % of cells showing positive fluorescence, respectively.

n.d. = not done.

region of the cytoplasm when immune sera against viral envelope and against the whole virus were used (Figs 1 and 2). Immune serum against viral nucleocapsid revealed viral antigen in the form of granules in the vicinity of the nuclear membrane but the number of granules was only about half that revealed by the other two sera just mentioned. Viral antigen in the nuclei of PK cells was not demonstrated by any of the three immune sera.

In PRV-infected PK cells viral antigen was demonstrated at all intervals tested in both the nuclei and cytoplasm in the form of bright granular and diffuse fluorescence. PRV strains TOP and TK-900 behaved similarly to each other.

XTC-2 cells infected with the two HSV-1 strains were examined after incubation at 30 and 36 °C. Viral antigen was demonstrated in the cell nuclei and cytoplasm in the form of granular and diffuse fluorescence as early as 16 hr after inoculation (p. i.). Immune serum against nucleocapsids revealed HSV-1 antigen in the nuclei and around the nuclear membrane while immune serum against viral envelope did so in the cytoplasm and around the nuclear membrane (Figs 3 and 4). Less viral antigen was demonstrated in cells grown at 30 °C than in those grown at 36 °C. Immunofluorescence failed to reveal PRV antigens in XTC-2 cells. The results of immunofluorescence studies are summarized in Table 4.

Table 5. Adsorption of HSV-1 and PRV on to PK and XTC-2 cells

Cell line	Virus	0 min	PFU/ml of virus adsorbed after			
			30 min	60 min	90 min	120 min
PK	HSV-1	2.4×10^6	7.0×10^5	6.4×10^5	4.0×10^5	2.5×10^5
XTC-2	HSV-1	7.4×10^6	1.8×10^6	1.5×10^6	1.5×10^6	1.1×10^6
	PRV	4.1×10^6	4.0×10^6	2.3×10^6	8.6×10^5	7.0×10^5

Table 6. Attempts at inducing persistent HSV-1 infection in PK and XTC-2 cells

Cell line	HSV-1 strain	Virus demonstrated on day	No. of subpassages of infected cells
PK	HSZP	8	1
		32	6
		12	2
	MA	10	2
		38	7
		14	2
		45	8
		14	2
XTC-2	HSZP	2	0
	MA	2	0
		7	1
		3	0

Adsorption of HSV-1 and PRV on to PK and XTC-2 cells

In spite of the low replication of HSV-1 in PK and XTC-2 cells and of PRV in XTC-2 cells both herpesviruses were adsorbed on to the little susceptible cells (Table 5). The amounts of virus adsorbed on to XTC-2 cells at 30 °C were 75.7% of HSV-1 within 30 min and 79.1% of PRV but only after 90 min. The amounts of HSV-1 adsorbed on to PK cells were 71.0 and 89.6% after 30 and 120 min at 36 °C respectively.

Cytomorphological changes in infected PK and XTC-2 cells

HSV-1-infected PK cells showed no marked changes. Alterations, mainly in the cell nuclei, were found in occasional foci in the cell monolayers. We observed a marked margination of chromatin on the nuclear membrane increased basophilia of the nucleoplasm and decay of the nucleoli into small basophilic formations. Degenerative changes in the cytoplasm were little marked, only in some cells was the cytoplasm partially destroyed.

Marked specific morphological changes occurred in XTC-2 cells infected with either HSV-1 strain tested. The first changes were observed in the nuclei, namely strong margination of chromatin on the nuclear membrane decay of nucleoli into small basophilic formations subsequently disappearing from the nuclei. At later stages of infection (24–48 hr p. i.) degenerative changes appeared in the cytoplasm. Characteristic polykaryocytes were formed in the cell monolayers, mainly in those infected with strain HSZP. The changes were less marked in cells grown at 30 °C.

Persistent infection of cell cultures with HSV-1

Persistent HSV-1 infection could not be induced in XTC-2 cells (Table 6). The latter were grown in 300-ml Roux bottles and inoculated with 10^7 TCID₅₀ of virus. No virus was demonstrated even in the 1st subpassage of

these cells with the exception of one experiment in which ICV (but not ECV) was found in the 1st subpassage in a titre of 1.5 log TCID₅₀/ml. Cells which no more contained infectious HSV-1 multiplied intensively and could be continuously passaged at 30 °C.

Persistent HSV-1 infection could be induced in PK cells; it was of various duration. The number of subpassages following primary infection varied depending on the duration of persistent infection. The titres of ICV and ECV were usually very low, varying from 1 to 3 log TCID₅₀/ml. Primary infection was induced in PK cells grown in 300-ml Roux bottles and inoculated with 10⁶–10⁷ TCID₅₀ of virus. In PK cell cultures infected with strain MA in which infectious virus was demonstrable up to the 8th subpassage on day 45 neither ICV nor ECV were detected in subpassages 2–4; subsequently ICV (but not ECV) was detected again in a titre of 1 log TCID₅₀/ml. Cells which no more contained infectious HSV-1 multiplied further and could be continuously passaged at 36 °C; they were susceptible to HSV-1 or PRV infection like normal uninfected PK cells. Indirect immunofluorescence failed to reveal HSV-1 antigens in the persistently infected PK cells.

Discussion

At present two stages of virus-infected cells are distinguished: productive and non-productive infection, characterized by a production of virus progeny and by no production of either infectious or non-infectious virus progeny, respectively. Abortive and restrictive infection in which small amounts of virus progeny or HSV structural components are produced (Roizman, 1972) are distinguished from the non-productive infection. XTC-2 cells represented a nonpermissive system for PRV, since reproduction of this *Herpesvirus* species was not demonstrated in them. In spite of that PRV virions were adsorbed on to XTC-2 cells, the virions (or their DNA) did not penetrate into the cells or the enzymatic mechanisms of XTC-2 cells did not allow the synthesis of infectious PRV or of its components.

Restrictive reproduction of HSV-1 was observed in PK and XTC-2 cells. Synthesis of both ICV and ECV was limited. More infectious virus was produced at high MOI, although the possibility cannot be excluded that the virus yield depended on the amount of HSV-producing cells. Abortive or restrictive HSV infection was observed in dog kidney cells (Aurelian and Roizman, 1964), CEC (Lowry *et al.*, 1971), hamster cells (HSV type 2; Docherty *et al.*, 1972), human embryo lung cells (Darai and Munk, 1973) and XC cells derived from rat sarcoma induced by Rous sarcoma virus (both HSV types; Docherty *et al.*, 1973).

The present experiments showed that XTC-2 cells are more susceptible to HSV-1 infection than PK cells. Also the removal of ICS from the growth medium after infection of the cells with HSV-1 led to a slight increase in the synthesis of infectious virus.

HSV-1 and PRV possess a broad range of susceptible cells *in vitro* and replicate practically in the same cell species. When comparing the different

susceptibility of cells from the rabbit, pig and frog from the point of view of evolution, it should be taken into account that on the phylogenetic tree constructed based on differences in the primary structure of cytochrome *c* in different organisms there is no significant difference between pairs frog — rabbit and frog — pig (11 amino acid exchanges in the polypeptide chain) while the difference between these pairs and the pair rabbit — pig is significant (4 amino acid exchanges; Dayhoff, 1969). From this point of view the difference between HSV-1 replication in rabbit cells (to which it had been adapted) and that in pig cells was significant but at the same time surprising.

Using indirect immunofluorescence we demonstrated HSV-1 antigens by monospecific sera against the nucleocapsid and viral envelope only around the nuclear membrane and in the paranuclear area of the cytoplasm of PK cells. This result was obtained mainly at high MOI. Viral antigen was never demonstrated in the nuclei. We also observed that at 72 hr p. i. the amount of viral antigens in the cells was lower. These findings suggest that, in this cell system, abortive or restrictive infection of a little susceptible cell system was involved, in which certain viral proteins (antigens) probably are not synthesized. This conclusion was supported by the results concerning the synthesis of infectious virus in PK cells. By contrast, this cell system proved to be fully susceptible to PRV infection.

In XTC-2 cells, HSV-1 antigen was demonstrated by immunofluorescence in both the nuclei and cytoplasm at both temperatures tested. The difference between 30 and 36 °C was that the amount of viral antigens at the former temperature was lower. These results showed that in XTC-2 cells also their nuclei were partially involved in replication of infectious virus and synthesis of viral antigens.

Cytomorphological observations on PK and XTC-2 cells infected with either HSV-1 strain were in accordance with the results obtained by immunofluorescence. While in PK cells little marked herpetic changes involving mainly the nuclei occurred in occasional foci in the monolayer of infected cells, marked herpetic changes were seen in both the nuclei and cytoplasm of XTC-2 cells. Acidophilic inclusions, however, were not observed in either cell system.

On serial passaging of undiluted HSV-1 and HSV-2, DIP are formed (Bronson *et al.*, 1973). We observed this phenomenon in the susceptible ZP cells infected at a high MOI with HSV-1. We carried out no serial passages.

We were able to induce persistent infection only with HSV-1 in PK cells. But as distinct from persistent herpetic infection in the presence of immune human serum (Szántó, 1963) or in the absence of antibody to HSV-1 and HSV-2 (Szántó, 1976), the persistent HSV-1 infection of PK cells was of limited duration. Thereafter, neither infectious HSV-1 nor its antigens were demonstrated and the cells continued to multiply. It was of interest that in some passages infectious HSV-1 was not detectable, although after a few further passages it reappeared again.

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Explanation of Micrographs (Plate XVIII):

- Fig. 1.* PK cells infected with HSV-1 strain HSZP, 24 hr p. i. Granular fluorescence of viral antigen on the nuclear membranes. $\times 200$.
- Fig. 2.* PK cells infected with HSV-1 strain MA, 48 hr p. i. Granular fluorescence around the nuclear membrane and diffuse fluorescence around the nuclear membrane and diffuse fluorescence in the paranuclear area of the cytoplasm. $\times 200$.
- Fig. 3.* XTC-2 cells infected with HSV-1, 24 hr p. i. at 36 °C. Granular and diffuse fluorescence of viral antigen. $\times 200$.
- Fig. 4.* XTC-2 cells infected with HSV-1, 48 hr p. i. Marked granular and diffuse fluorescence in the majority of cells. $\times 200$.