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RESTRICTED REPLICATION OF HERPES SIMPLEX VIRUS TYPE 1 IN PIG KIDNEY CELLS

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Summary. — Pig kidney (PK) cells infected with herpes simplex virus type 1 (HSV-1) strains HSZP and RO, respectively, synthesized low amounts of infectious virus; virus-specific antigens detected by immunofluorescence (IF) staining were seen only in a small proportion of cells. When propagated in 2—4 subsequent passages, PK cells infected with the HSZP strain (PK--HSZP cells) became free of infectious virus and virus-specific antigens as detected by IF staining. In contrast, PK cells infected with the RO strain (PK-RO cells) permanently harboured the infectious virus and virus-specific antigens in the course of 10 subsequent passages. The latter were detectable 48—72 hr after seeding. The constantly low virus yields were significantly enhanced when restrictive PK cells were mixed with permissive ZP cells. Cocultivation of variable amount of PK cells (at the 2nd, 5th and 7th passages) with a constant number of ZP cells showed that 10^2—10^3 PK cells were necessary to transfer the HSV to indicator cells. Immediate early and early HSV-1 coded antigens were seen by IF in the nuclei of a small proportion of PK-RO cells; similarly, virion and nucleocapsid antigens were found in the nuclei and cytoplasm of a few cells of the PK-RO monolayers.

Key words: *herpes simplex virus type 1*; *immunofluorescence*; *PK cells*; *restrictive persistent infection*; *cocultivation*

Introduction

PK and XTC-2 (Xenopus laevis frog) cells have been found restrictive for HSV-1 as followed by infectivity assay, IF staining and electron microscopy (Szántó *et al.*, 1980; Čiampor and Szántó, 1982). For a limited period, persistent infection has been established in PK cells infected with selected HSV-1 strains. This form of persistence was clearly different from that established in permissive cells in the presence of antibodies (Szántó, 1963). Our attention has been focused on restricted growth of selected HSV-1 strains — especially strain HSZP and strain RO — occurring in a small

proportion of PK cells infected at multiplicity of infection (m.o.i.) 1 or more PFU per cell. Under such conditions, the synthesis of infectious virus, nonstructural and structural virus-coded proteins as detected by IF staining was compared in restrictive PK cells, in mixed cultures of PK and ZP cells and in permissive ZP cell cultures.

Materials and Methods

Viruses. Strains HSZP and RO of HSV-1 were grown in ZP cells (rabbit lung fibroblasts) as previously described (Szántó, 1960; Szántó *et al.*, 1972). Infected monolayers were incubated for 48 hr at 36 °C in Earle's medium containing 0.5% lactalbumin hydrolysate (LAH), 0.1% yestolate, 0.45% glucose, 5% inactivated calf serum (ICS) and antibotics. After three times freezing and thawing, cells were harvested into a small amount (4 ml per 1000 ml flask) of distilled water and stored at —70 °C. Stock strains titered 3×10^7 and 1×10^8 PFU/ml, respectively.

Cells. Pig kidney (PK) cells were grown in medium Epl (Michl, 1962) containing two calf serum protein fractions (0.375% albumin and 0.025% alpha globulin) and 0.2% LAH supplemented with 5% ICS and antbiotics. The same medium was used also for cocultivation experiments to create standardized growth conditions for both cell lines.

Infection of PK cells. Three types of cell cultures were infected (1) PK cells grown in tubes or on cover slips (1×10^5 cells/ml); (2) PK cells mixed with ZP cells before seeding (5×10^4/ml each); (3) ZP cells (1×10^5/ml). After incubation for 48 hr, cultures were infected either with the HSZP strain or with the RO strain at m.o.i. of 1 PFU/cell. Following 60 min adsorption, cells were washed 3 times in phosphate buffered saline and replenished with nutrition medium.

Persistent infection of PK cells. Cultures seeded in Roux bottles were infected with HSZP or RO strains at m.o.i. of 1 PFU/cell. By 48 hr, cells were trypsinized (0.25% trypsin and 0.02% sodium EDTA) and further propagated (designated as PK-HSZP or PK-RO cells, respectively). At different passage levels, test tubes with or without cover slips were seeded either with the persistently infected PK cells (1×10^5/ml) or with a mixture of these cells with the permissive ZP cells (5×10^4/ml of each). All passages were done by 7 days intervals the first passage being the only exception.

Evaluation of the number of cells producing infectious virus. A variable number (1×10^2, 1×10^3, 1×10^4 and 5×10^4) of PK-HSZP or PK-RO cells was added at different passage levels (1st, 2nd, 4th or 1st, 2nd, 5th, 7th respectively) to a constant number of ZP cells (1×10^5/ml). The mixtures were seeded into test tubes with or without cover slips and incubated for intervals shown in Tables. The amount of infectious virus or the number of cells showing positive IF was evaluated.

Assay of infectious virus. Cells and nutrition fluid were frozen and thawed, plated as whole onto monolayers of ZP cells in Leighton tubes and incubated in the presence of 0.5% methylcellulose overlay. Plaques were counted after 4 days incubation.

Sera. Semipurified HSV-1 virions were prepared from sonicated infected ZP cells resuspended in distilled water and centrifuged (68,000 × g for 45 min) in a 10—30% Ficoll gradient (Matis *et al.*, 1975). Antivirion serum was raised in rabbits by 3 injections (100 μg protein each) of the semipurified virion material mixed with incomplete Freund's adjuvant. Purified nucleocapsids were prepared by centrifugation for 6 hr at 168,000 × g in 5—55% saccharose gradient dissolved in D_2O (Matis *et al.*, 1975; Leššo *et al.*, 1976). Anti-nucleocapsid serum was prepared in rabbits as described above using 50 μg nucleocapsid protein per injection. Serum to immediate early and early proteins was prepared by 5 subsequent intramuscular injections of rabbits with an antigen extract from SIRC cells treated with cycloheximide (100 μg/ml) for 5 hr post infection (p.i.) and then harvested after 4 hr incubation at 37 °C (Matis and Rajčáni, 1980). In parallel, a similarly prepared antigen extract labelled with ^{14}C-amino acids (370 kBq/ml) was tested against the antiserum and control (preimmune) serum. The following nonstructural HSV-1 coded proteins synthesized from 2—4 hr p.i. were identified by electrophoresis of the immune precipitate in polyacrylamide gel: 175 K, 146 K, 132 K, 110 K, 87 K, 44 K and 32 K. All sera were adsorbed to ZP cells, SIRC cells and to PK cells before staining.

Immunofluorescent staining. Cover slips were washed in buffered saline, air dried, fixed in acetone and stained either by the indirect IF method or by the anticomplement IF staining

(ACIF). For indirect staining sera diluted 1 : 10 were incubated with the monolayers followed by the conjugate SwAR (Sevac, Prague) diluted 1 : 5. ACIF staining was performed as follows: after 40 min incubation with the serum diluted 1 : 10, cover slips were washed, incubated with complement (2 U/0.1 ml), washed and stained subsequently with 2 conjugates, namely GAC3 (Dynatech, Switzerland) and SwAR. Cover slips mounted into Elvanol were viewed in Fluoval microscope (C. Zeiss, Jena, G.D.R.) and photographed on roentgen fluorapid film (RS-2, ORWO G.D.R.).

Results

Infectivity assay and IF in PK cells

PK cells infected with the two HSV-1 strains at m.o.i. 1 PFU/cell replicated the virus to low titres (Table 1). Virion antigens as detected by indirect IF were seen in single cells infected with the HSZP strain (48—72 hr p.i.) and in less than 1% of cells infected with the RO strain (24—48 hr p.i.). In contrast, infected ZP cells yielded large amounts of infectious virus and virion antigens were present in a great majority of these cells. Alternatively, monolayers prepared by seeding of a mixture of PK and ZP cells were highly permissive for the RO strain. When infected with the HSZP strain, the mixed cultures showed virion antigens by 24 hr in 40% of cells, by 72 hr in 80—90% of cells (Fig. 1), respectively. Virus antigen was seen in the cytoplasm and nuclei of cocultivated cells stained with anti-virion serum and predominantly in the nuclei of these cells stained with anti-nucleocapsid serum. The number of cells displaying positive staining with the latter serum was about the half of those stained with anti-virion serum. In PK cells stained with anti-virion serum the virus coded antigens were seen predomidominantly in perinuclear area of the cytoplasm of a small proportion of infected cells (Fig. 2). By ACIF staining anti-virion serum detected the nuclear antigens as well.

Serum against early and immediate early proteins stained the nonstructural polypeptides in the nuclei of single PK cells by 24—48 hr p.i. (Fig. 3). The number of fluorescing nuclei was higher in PK cells infected with the RO strain than in those infected with the HSZP strain. In the former, bright

Table 1. Infectivity assay and IF in PK cells, ZP cells and mixed cultures of PK with ZP cells

HSV strain	Cells	Interval after seeding 24 hr Virus titre	IF	48 hr Virus titre	IF	72 hr Virus titre	IF
	PK	5×10^2	0	3.1×10^2	±	5×10^2	±
HSZP	PK+ZP	5×10^2	++	1×10^5	+++	2×10^5	+++
	ZP	2×10^4	+++	5×10^6	+++	1×10^6	+++
	PK	5×10^2	±	3.1×10^3	±	n.d.	n.d.
RO	PK+ZP	2×10^7	++	5×10^5	+++	n.d.	n.d.
	ZP	2×10^7	+++	2×10^5	+++	n.d.	n.d.

n.d. = not done; 0 = negative; ± = IF in single cells (< 1%); + = IF in 5—10% of cells; ++ = IF in 20—50% of cells; +++ = IF in 60—100% of cells.

Table 2. Infectivity assay and IF in persistently infected PK cells propagated for 10 passages

Cells	No. of passages	Interval after seeding (hr) 24 Virus titre	24 IF	48 Virus titre	48 IF	72 Virus titre	72 IF
PK-HSZP	1st	n.d.	0	5×10	0	3.1×10	0
PK-HSZP+ZP		n.d.	n.d.	5×10^5	++	5×10^6	+++
PK-HSZP	2nd	0	0	0	0	0	0
P-HSZP+ZP		0	0	0	0	0	0
PK-RO	1st	n.d.	0	5×10^4	+	1×10^5	++
PK-RO+ZP		n.d.	±	5×10^5	++	2×10^6	+++
PK-RO	4th	1×10^4	+	1×10^4	+	2×10^4	++
PK-RO+ZP		2×10^5	+++	5×10^5	++	5×10^5	+++
PK-RO	10th	n.d.	n.d.	3.1×10^2	+	2×10^2	+
PK-RO+ZP		n.d.	n.d.	2×10^6	++	5×10^5	+++

Legend in Table 1.

granular fluorescence was seen dispersed in the nuclei or at the inner nuclear membrane (Fig. 4).

Infectivity assay and IF staining in persistent infection of PK cells

The results of infectivity assay and IF staining of PK cells in the course of persistant infection are shown in Table 2. Neither infectious virus nor the viral antigen were found in PK-HSZP cells in the 2nd passage, though in the 1st passage traces of infectious virus were detected by 48-72 hr after seeding. In contrast, persistent infection had been clearly established in PK-RO cells as confirmed in the 1st 4th and 10th passages. Virion antigens were found by IF staining in 5—20% of cells at different intervals after seeding (Fig. 5). When persistently infected PK cells were mixed with ZP cells and incubated for 48—72 hr, the virus yields increased considerably and the number of cells showing virus antigens became also abundant. If the PK-RO cells were stained with the anti-nucleocapsid serum, the number of fluorescent cells was aproximately the half of those stained with the anti--virion serum. Thus, the results of IF staining confirmed the restricted replication of the RO strain in the propagated PK cells (Fig. 6).

Evidence for restricted growth of HSV-1 in PK cells

Various amounts of PK-RO or PK-HSZP cells were mixed with a constant number of indicator cells at different passage levels. Table 3 shows that 10^3 PK-RO cells regularly transfered the infectious virus to ZP cells whereas 10^2 PK-RO cells did so in the 2nd and 7th passages. The ability of PK-RO cells to transfer virus into indicator cells was dependent on their concentration. In the 4th passage no infectious progeny was present even in 5×10^4 PK--HSZP cells, while in the 1st passage already 10^2 cells had been infectious (Table 4). The latter results confirmed the different behaviour of RO and HSZP strains in restrictive PK cells, which have turned out less permissive in relation to the HSZP strain as compared to the RO strain.

Table 3. Infectivity assay and IF in persistently infected PK-RO cells cocultivated with ZP cells

Cells	Virus detection							
	1st passage		2nd passage		5th passage		7th passage	
	Virus titre	IF	Virus titre	IF	Virus titre	IF	Virus titre	IF
PK 1×10^5+ZP 1×10^5	3.1×10^4	+++	1×10^4	+	5×10^3	++	3.1×10^3	+
PK 1×10^2+ZP 1×10^5	1.6×10^3	++	0	0	0	0	1×10^4	+
PK 1×10^3	5×10^5	+++	1×10^2	+	1×10^3	+	2×10^4	+++
PK 1×10^4	3.1×10^5	+++	1×10^4	++	5×10^5	+++	5×10^5	+++
PK 5×10^4	1.6×10^4	+++	5×10^5	+++	3.1×10^5	CPE	2×10^6	+++

Legend in Table 1.
CPE = cytopathic effect.

Table 4. Infectivity assay and IF in persistently infected PK-HSZP cells cocultivated with ZP cells

Cells	Virus detection 1st passage Virus titre	IF	2nd passage Virus titre	IF	4th passage Virus titre	IF
PK 1×10^5 + ZP − 0	1×10^3	±	0	0	0	0
PK 1×10^2 + ZP 1×10^5	1×10^5	+++	0	0	n.d.	0
1×10^3 1×10^5	2×10^5	+++	0	0	n.d.	0
1×10^4 1×10^5	2×10^6	CPE	3.1×10	0	0	0
5×10^4 1×10^5	3.1×10^5	CPE	2×10^3	+	0	0

Legend on Table 1 and Table 3.

Discussion

Latent infection of regional sensory ganglia with HSV-1 has been confirmed in experimental animals by many investigators and there is no doubt that it frequently occurs also in man (Stevens and Cook, 1971; Stevens et al., 1972; Benda *et al.*, 1973; Baringer, 1975; Rajčáni *et al.*, 1975; Stevens, 1975). However, the molecular basis for HSV latency is still not fully elucidated. Controversy concerns the transcription of virus-specific mRNA during latency as there is not clear whether certain virus-coded proteins are continuously produced in a small proportion of ganglion cells (Puga *et al.*, 1978; Galloway *et al.*, 1979, 1982; Rajčáni and Matis, 1981).

These mechanisms are supposed to be investigated more efficiently in cell culture model systems. The main features of restrictive HSV replication described so far in vitro are as follows: impaired penetration of the virus into infected cells, decreased or inhibited DNA synthesis, insufficient production of viral glycoproteins or impaired envelopment at the nuclear membrane (Aurelian and Roizman, 1964; Docherty *et al.*, 1972, 1973; Bronson *et al.*, 1973; Darai and Munk, 1973; Campadelli-Fiume *et al.*, 1980). We reported restrictive growth of HSV and limited production of virus antigens in PK and XTC-2 cells (Szántó *et al.*, 1980). In PK cells the penetration of adsorbed virions was impaired and, in addition, the envelopment of nucleocapsids at the nuclear membrane was hampered (Čiampor and Szántó, 1982). The present work has shown that replication of HSV is limited to a small proportion of cells and that addition of ZP cells leads to enhanced virus growth in mixed cultures. By means of 3 different antisera (anti-virion, anti-nucleocapsid, serum to non-structural proteins) the synthesis of structura land non-structural virus coded antigens was demonstrated in a proportion of infected PK cells. Indirect IF staining with anti-virion serum has detected predominantly cytoplasmic antigens (Szántó *et al.*, 1980). Nuclear fluorescence of structural capsid antigens and of non-structural virus-coded antigens was found by ACIF staining (Figs 3—4). These antigens revealed a faint specific fluorescence only when detected by indirect IF staining. A brilliant nuclear

staining was found in PK cells stained with anti-virion serum by ACIF as compared to invariably occurring nuclear staining with this serum by indirect method.

The long-term propagation of HSV infected PK cells showed that persistent infection had been established only with RO strain providing that cells were subsequently propagated for 4—10 passages. Virus-specific antigens were seen in 5—20% of the persistently infected cells and their number varied in different passages. In contrast to PK-RO cells, the PK-HSZP cells turned out negative for both infectious virus and viral antigens. The virus persisting in PK-RO cells replicated well in ZP cells as indicated by 3—4 $\log_{10}$ increase of its infectious titre and by 6- to 10-fold increase of the number of fluorescing cells in mixed cultures. Comparing the staining of mixed cells with the anti-nucleocapsid serum to their staining with the anti-virion serum, about 50% less cells were positive with the former than with the latter. This could be explained by enhanced envelope antigen formation under the permissive conditions. When a varying number of persistently infected PK cells was mixed with a constant number of indicator cells, the amount of transfered infectious virus units was dependent on the number of transfered PK cells. PK-HSZP cells did not transfer any infectious virus.

O'Neill (1977) found no production of infectious virus in human embryonic fibroblasts treated with ara-C and shifted up to 39.5 °C after ara-C reversal. If cells were not shifted to the elevated temperature or if the elevated temperature was reduced again, infectious virus production had been reactivated in a small proportion of cells. In response to an another inhibitor of viral DNA synthesis, 1-3-d-arabino-furanosylcytosine, and, due to rise of the cultivation temperature to 40.5 °C, the replication of HSV-1 in human embryonic cells was completely inhibited. The virus synthesis was attained by decreasing the incubation temperature or by superinfecting with another herpesvirus (Wigdahl *et al.*, 1981), implicating the involvement of an early herpesvirus coded function in the regulation of virus replication.

The restrictive virus-cell interactions described in our cell system seemed to depend on properties of both, the virus strain and the individual cells in the monolayer population.

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Explanation of Micrographs (Plates XIV—XV):

Fig. 1. Mixed culture of PK and ZP cells, growth of the RO-HSV-1 strain. Viral antigen in the nucleus, at the nuclear membrane and in the cytoplasm. Indirect IF staining, anti-virion serum, × 120.

Fig. 2. PK cells 24 hr after infection with the RO strain. Positive fluorescence at the nuclear membrane and diffuse fluorescence in the cytoplasm of a single cell. Indirect IF staining anti-virion serum, × 200.

Fig. 3. PK cells 48 hr after infection with the RO strain. Predominantly intranuclear staining with the serum to immediate early and early antigens; ACIF method, × 360.

Fig. 4. A focus of PK cells infected with the RO strain, granular nuclear and diffuse cytoplasmic fluorescence with the anti-virion serum, ACIF method, × 160.

Fig. 5. PK-RO cells in their 4th passage, 48 hr after seeding. A part of cells show diffuse staining of cytoplasm. Anti-virion serum, indirect IF, × 160.

Fig. 6. PK-RO cells in their 4th passage were mixed with ZP cells and seeded; 48 hr later, widespread staining in cytoplasm of nearly each cell can be seen. Indirect IF, × 160.