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SCANNING ELECTRON MICROSCOPY OF CV-1 AND HeLa CELLS INFECTED WITH HERPES SIMPLEX VIRUSES TYPES 1 AND 2

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Summary. — Production of virus particles in CV-1 or HeLa BU-25 cells was investigated after their infection with several strains of herpes simplex virus (HSV) types 1 and 2, especially in relation to the association of virus particles with cells, the ratio of plaque forming units (PFU) to the whole number of virus particles and the morphological characteristics of cytopathic effect (CPE). Growth curves of the viruses differed according to the combination of cells and infecting virus strains. At 20 hr p.i., the number of cell-associated or cell-free viruses ranged from 5×10^8 to 5×10^9 PFU/35 mm dish or from 5×10^2 to 1.5×10^4 PFU/cell irrelevant of virus serotype or the morphology of CPE. In the case of CV-1 cells, the ratios of the number of the virus particles to PFU ranged from 100 to 640 and/or 18 to 110, respectively, depending on the CPE of rounding type or of fusion type. In case of CPE of fusion type, a higher rate of infectious particles was observed.

Key words: HSV-infection; cytopathic effect; scanning electron microscopy; particle count

Introduction

After infection of the cells with HSV, they exhibit different morphology of CPE according to the combination of cell line and virus strain (Nii, 1961; Ejercito *et al.*, 1968). This means that interactions between cells infected with HSV are governed by multiple factors, both cellular and viral (Keller *et al.*, 1970; Manservigi *et al.*, 1977; Lee and Spear, 1980; Bzik and Person, 1981). In our previous paper, we reported on sequential events occurring since production to release of progeny virus particles in CV-1 cells infected with HSV type 1 (strain HF) which showed CPE of fusion type (Katsumoto *et al.*, 1981*b*). In this paper the relationship between morphological characteristics of the CPE and production and/or cell-association of virus particles was investigated by scanning electron microscopy, *in situ* thin section electron microscopy, virus particle counts and estimation of plaque forming units.

Materials and Methods

Cells. CV-1 (an African green monkey kidney cell line) and HeLa BU-25 cells (Kit and Leung, 1974) were used. As the culture medium, Eagle's minimal essential medium (MEM) (Nissui,

Tokyo) supplemented with 10% newborn calf serum (Microbiological Associates, Waldersville, Md., U.S.A.) was used. As vessels for cell culture, 35 mm Falcon plastic Petri dishes were used.

Viruses. HSV type 1 (strains HF and WT20) and type 2 (strains UW268 and NGG) were used throughout the experiments, and their infectivity was checked by their plaque formation in CV-1 monolayers. Cells were infected with the virus at the input multiplicity of 1 plaque forming unit (PFU)/cell.

Electron microscopy. Electron microscopical procedures employed were: in situ thin sections (TS), scanning electron microscopy (SEM) and virus particle counts.

For TS, cells grown as monolayers on plastic Petri dishes were fixed with 3% glutaraldehyde and 1% OsO_4 and embedded *in situ* into Epon 812 (Abercrombie *et al.*, 1971; Brunk *et al.*, 1971). Sections made vertically to the substrate were stained with uranyl acetate and lead citrate.

For SEM, cells grown on converslips placed into plastic Petri dishes were fixed as in case of TS and then were treated with tannic acid (Katsumoto *et al.*, 1981*a*). The specimens were dehydrated through a graded series of ethanol and then dried using the critical point drying method. The dried samples were coated with metals in a vacuum evaporator.

Particle counts were carried out by the loop drop method using phosphotungstate to provide negative contrast (Watson *et al.*, 1963, 1964). At the times indicated, infected cells were scraped off with a rubber policeman and the cell suspension in the culture fluid was centrifuged at 2,000 rev/min for 10 min. The supernatant was used for the assay of cell-free viruses. The cell pellet was resuspended in fresh culture medium, freezed-thawed for three times and centrifuged as above to get the cell-associated virus.

For observation of TS and particle counts, the Hitachi HU-12A electron microscope and for SEM the Hitachi S-430 seanning electron microscope were used.

Results

Morphology of HSV CPE on CV-1 cells and the appearance of progeny virus particles

After infection of CV-1 cells with one of the four HSV strains, infected cells were observed by electron microscopy at 6, 7, 8, 9 and 20 hr p.i. When CV-1 cells were infected with HSV type 1 WT20, virus particles appeared on the cell surface at 8 hr p.i., but the majority of them was found in the nuclei or in the cytoplasm. At 20 hr p.i., almost all the cells were rounded, many virus particles were present on the surface of the half of the cells attached to the substrate (Fig. 2); often many virus particles were observed in vacuoles of the rounded cells (Fig. 8). On the other hand, CV-1 cells infected with HSV type 1 strain HF exhibited CPE of the fusion type since 9 hr p.i. showing a partial fusion with neighbouring cells. At 8 hr p.i., the progeny virus particles were mainly present in the nuclei and in the cytoplasm and a small number of virus particles was present on the cell surface only. At 20 hr p.i., extensive cell fusion occurred resulting in formation of polykaryocytes, and the number of microvilli-like projections increased; numerous virus particles were observed on the cell surface (Figs 3 and 4).

CPE of CV-1 cells infected with type 2 strain UW268 was of rounding type. At 20 hr p.i., virus particles were rarely present on the cell surface (Fig. 5). Inside of the rounded cells, single virus particles or their aggregates were often seen within vacuoles (Fig. 9). CV-1 cells infected with type 2 NGG exhibited CPE of the fusion type at 7 hr p.i., but virus particles appeared on the cell surface already at 6 hr p.i. At 9 hr p.i., polykaryocyte formation as the result of cell fusion and increase of the number of the microvilli-like

Table 1. Morphology of CPE and association of HSV with the cell surface

Virus		Morphology of CPE (cell associated virions)			
Type	Strain	CV-1		HeLa BU-25	
1	WT20	r	+	r	±
	HF	f	++	r	+
2	UW268	r	±	r	±
	NGG	f	+	r	±

Virus particles on the cell surface: ±: seldom, +: small number, ++: many
Cells infected with 1 PFU/cell, harvested 20 hr p.i.
r: CPE rounding type; f: CPE fusion type.

projections became predominant and many virus particles were attached to the cell surface. These findings were the most evident at 20 hr p.i. (Figs 6 and 7).

Morphology of HSV CPE in HeLa Bu-25 cells and the distribution of the progeny virus

HSV type 1 strains, WT20 and HF, and type 2 strains UW 268 and NGG, exhibited in HeLa BU-25 cells a CPE of the rounding type. As the result of CPE, WT20 induced loose aggregation of rounded cells at 20 hr p.i. and virus particles were seldomly observed on the cell surface (Fig. 11). At 20 hr p.i., strain HF induced rounding of the cells concomitant with small number of fusiform cells and many virus particles attached to cell surface (Figs 12 and 13) and inside the cells (Figs. 16 and 17).

Concerning the type 2 strains UW268 and NGG, although many virus particles could be observed inside of the rounded cells (Fig. 18 and 19), they could be seldom seen on the cell surface (Fig. 14 and 15) at 20 hr p.i.

The correlation among serotypes of the virus, the morphology of the CPE and the number of virus particles associated with the cell surface are sum-

Table 2. Association of virus progeny with the cells

Virus		Cells	CPE	Particles/Cell		PFU/Cell		Particles/PFU	
Type	Strains			CA	CF	CA	CF	CA	CF
1	WT20	CV-1	r	2,900	1,700	29	2.9	100	590
1	HF	CV-1	f	14,400	10,400	136	180	106	58
2	UW268	CV-1	r	2,700	1,570	4.2	6.7	640	230
2	NGG	CV-1	f	1,900	3,200	90	180	21	18
1	HF HeLa	BU-25	r	2.500	500	50	25	50	20
2	NGG HeLa	BU-25	r	2,100	750	63	13	33	58

CA = cell associated, CF = cell-free.
Cells infected with 1 PFU/cell and harvested at 20 hr p.i.

marized in Table 1. The tendency to attach to the cell surface was stronger in case of type 1 than type 2 HSV strains.

Cell-associated and cell-free infectivity and physical particle counts of HSV types 1 and 2

Virus growth curves differed from each other according to the combinations of the virus strains and cells. In Table 2, results of the determination of progeny virus infectivity and particle counts are summarized at 20 hr p.i. As for CV-1 cells, yields of infectious virus were lower in the case of CPE of the rounding type than at fusion type. Strains HF and NGG induced CPE of fusion type in CV-1 and a CPE of the rounding type in HeLa BU-25 cells. These two strains produced higher yields of infectious virus in the CV-1 than in the HeLa BU-25 cells.

Discussion

The multiple factors, cellular and viral, affecting morphological expression of CPE has been discussed by several investigators (Ejercito *et al.*, 1968; Haffey and Spear, 1980; Lee and Spear, 1980). For cell fusion to occur, the expression of HSV gene product(s) is necessary (Manservigi *et al.*, 1977; Ruyechan *et al.*, 1979). It is of interest to see correlation between morphological characteristics, fusion or rounding, and egression or release of the progeny virus. In our previous paper, we reported the usefulness of in situ thin section (TS) and scanning electron microscopy (SEM) for the analysis of the progeny virus release (Katsumoto *et al.*, 1981*b*). Employing this technology, the association of HSV with cell membrane or other parts of the cells was anallysed.

Irrelevant to the morphological characteristics of the CPE induced by HSV types 1 and 2, association of progeny virus with infected cell membrane tended to be stronger with type 1 than with type 2. This fact may be explained by a type-specific glycoprotein involved in the attachment of HSV type 1 to the cell membrane as suggested by Vahlne *et al.* (1979). Sarmiento *et al.* (1979) reported that the viral fusion factor had a critical role in penetration the cell membrane. In our experiments, virus yields tended to be higher when CPE was of fusion type as compared with that of rounding type. The role of the viral and the cellular fusion factors on the effectivity of viral infection will be further studied. Regardless of the viral serotypes, in the case of CPE of the rounding type the progeny virus particles often remained within the cell vacuoles, either as single particles or as their aggregates. This fact may suggest the role of cytoskeleton in the transport of virus particles to cell surface.

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Explanation of Electron Micrographs (Plates LI—LIV):

Figs 1—7. CV-1 cells infected with HSV types 1 and 2 were examined by SEM at 20 hr p.i. When CV-1 cells were infected with HSV-1 (WT20) and HSV-2 (UW268) they exhibited CPE of rounding type, while CV-1 cells infected with HSV-1 (HF) and HSV-2 (NGG) exhibited CPE of fusion type. The cells were infected at input multiplicity of 1 PFU/cell.
1 — mock-infected, × 1,200; 2 — strain WT20, × 600 (insert × 4,500): 3 — strain HF, × 600; 4 — strain HF, × 4,500; 5 — strain UW268, × 600 (insert × 4,500); 6 — strain NGG, × 600; 7 — NGG, × 4,500.

Figs 8—9. In situ thin sections of CV-1 cells infected with HSV-1 (WT20) or HSV-2 (NGG) at 20 hr p.i.
CV-1 cells were infected at the input multiplicity of 1 PFU/cell with HSV-1 (WT20) or HSV-2 (UW268) and at 20 hr p.i. were fixed and processed for TS. Many virus particles were observed inside the infected cells. 8 — strain WT20, × 9,000; 9 — strain UW268, × 9,000.

Figs 10—15. Electron micrographs of HeLa BU-25 cells, as observed by SEM at 20 hr after infection of HeLa BU-25 cells with HSV types 1 and 2.
All virus strains, type 1 (WT20 and HF) and type 2 (UW268 and NGG), exhibited CPE of rounding type. HeLa BU-25 cells were infected at the input multiplicity of 1 PFU/cell and at 20 hr p.i. they were fixed and viewed in SEM.

10 — mock-infected, × 1,200; 11 — strain WT20, × 750; 12 — strain HF, × 750; 13 — strain HF, × 4,500; 14 — strain UW268, × 750; 15 — strain NGG, × 750.

Figs 16—19. In situ thin sections of HeLa BU-25 cells infected with HSV-1 (HF) or HSV-2 (NGG) at 20 hr p.i.

HeLa BU-25 cells were infected at the input multiplicity of 1 PFU/cell by HSV-1 (HF) or HSV-2 (NGG). At 20 hr p.i. they were fixed, processed and viewed in TS. Many virus particles were observed inside the cytoplasm.

16 — strain HF, × 9,000; 17 — strain HF, × 20,000; 18 — strain NGG, × 6,000; 19 — strain NGG, × 20,000.