In situ Electron Microscopical Observation of Cells Infected with Herpes Simplex Virus

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SUMMARY

Transport and release of herpes simplex virus (HSV) in an African green monkey kidney cell line (CV-1) was followed by electron microscopy for up to 24 h p.i. Transmission electron microscopy and scanning electron microscopy were employed. For the former approach, electron microscopical autoradiography of whole cultured cells and in situ thin section techniques were used. The following new observations were made. (1) Except in peripheral parts of the cells, where the cytoplasmic membrane could make a ruffling movement relatively freely, virus particles were found only on the dorsal surface of the cell and not on the surface facing the substratum. By observation of thin sections in situ, it was confirmed that the virus particles within intracytoplasmic vacuoles were apparently released by a reverse phagocytic process from the cell surface adjacent to microvillus projections. (2) Progeny virus particles in the nucleus moved to the cell surface within 2 h after maturation.

INTRODUCTION

The process of infection by herpes simplex virus (HSV) has been studied extensively. Margination of chromatin was observed first and subsequently the maturation process and release of the virion from infected cells (Epstein, 1962a, b; Holmes & Watson, 1963; Morgan et al., 1959, 1968; Nii et al., 1968; Nii, 1971; Schlehofer et al., 1979; Schwartz & Roizman, 1969a, b).

These observations were mainly performed on thin sections of the cells which were detached from the substratum, collected and fixed; it is probable that artificial changes occurred both to the surface of the cells and to their internal organization. In this paper we present data of in situ morphological studies of HSV-infected cells and add new data on the process of infection by HSV.

METHODS

Cells. An African green monkey kidney cell line, CV-1, was used for the study. For scanning electron microscopy (SEM), cells were grown on coverslips placed in Falcon plastic Petri dishes. For electron microscopical autoradiography (EMARG), cells were grown on nickel grids coated with formvar and carbon and placed in the Petri dishes. For the tissue culture medium, a 1:1 mixture of YLE medium and Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% newborn calf serum (Microbiological Associates, Walkersville, Md., U.S.A.) was used. The cultures were incubated in a CO2 incubator (5% CO2:95% air) at 36 °C. Cells grew similarly on a plastic substratum, on glass coverslips and on carbon-coated grids.
**Virus.** HSV type 1, strain HF, was used. Virus stock was made by infecting CV-1 cells and the infectious titre of the virus was measured on CV-1 cell monolayers by the plaque method. At the subconfluent stage, the cells were infected with the virus at an input m.o.i. of 14 p.f.u./cell.

**Electron microscopy.** Electron microscopical procedures employed were thin section (TS), electron microscopical autoradiography of whole cultured cells (EMARG) in situ and scanning electron microscopy (SEM). CV-1 cells, infected by HSV or mock-infected, were processed for electron microscopical observations at 2, 4, 6, 8 and 24 h post-infection (p.i.).

For TS, cells grown on plastic Petri dishes were fixed with 3% glutaraldehyde and 1% OsO₄ and were embedded in situ in Epon-812 (Abercrombie et al., 1971; Brunk et al., 1971). Sections were made vertically to the substratum and were stained by uranyl acetate and lead citrate. For EMARG, cells grown on grids were labelled with ³H-thymidine (20 μCi/ml) at 4 or 5 h after infection with HSV until 8 h p.i. At the end of the labelling period, cells were fixed with 3% glutaraldehyde and nuclear track emulsion (Sakura NR-H2 diluted 1:5 with water) was mounted on to the reverse surface of the grid as previously described (Katsumoto et al., 1980). Exposure time was 4 days at room temperature in a dried light-tight box. Alternatively, for pulse-labelling experiments, CV-1 cells were infected with HSV at an input multiplicity of 5 p.f.u./cell, labelled with ³H-thymidine (20 μCi/ml) for 20 min at 4.5 h p.i.
In situ analysis of HSV-infected cells

Fig. 2. An in situ thin section (a) and SEM (b) of non-infected CV-1 cells. In (a) S represents the substratum. Only one microvillus can be seen in area.

and chased in medium containing $1 \times 10^{-6}$ M unlabelled thymidine. These cells were processed for EMARG as mentioned above (except for an exposure time of 16 days), at 6, 7, 8 and 9 h p.i. For SEM, cells grown on coverslips were fixed in 3% glutaraldehyde and 1% OsO$_4$ and further processed employing a critical point drying method. For the observation of the samples, a Hitachi HU-12A electron microscope was used for TS and EMARG, and a Hitachi electron microscope type S-700 was used for SEM.

RESULTS

Until 4 h p.i., cells infected with HSV at 14 p.f.u./cell did not show any morphological changes and the cell surface was smooth except for the presence of a small number of
microvilli. At 6 h p.i. cell fusion between adjacent cells started and the number of microvillus projections on the cell surface increased. This tendency increased with time and at 8 h p.i. the density of projections on the cell surface became very high. At this time the electron micrograph of a thin section of infected cells in situ (Fig. 1a) showed intranuclear margination of chromatin and a lightened nucleus compared with a non-infected control (Fig. 2a). The
In situ analysis of HSV-infected cells

Fig. 5. Electron micrographs of CV-1 cells infected with HSV at 24 h p.i. (a) Scanning electron micrograph shows many particles of various sizes (80 to 200 nm) in solitary or in an aggregated state together with bleb-like structures. (b) By in situ thin section, filamentous structures (arrow) as well as many virions can be seen as at 8 h p.i.

surface of infected cells had many projections as observed by SEM (Fig. 3) and virus particles were observed on it as well as in the nucleus, but on the cell surface facing the substratum no virus particles were found (Fig. 1a). Mock-infected cells showed fewer surface projections (Fig. 2b) than infected cells (Fig. 3). Concerning pseudopodia or the periphery of the cell, where the cell membrane and the cytoplasm move freely, virus particles can also be observed in the interspace between pseudopodia and the substratum (Fig. 1b). The status of infected cell—substratum adhesion did not change up to 8 h p.i. (Fig. 1a) and the cell attached to the substratum, apparently leaving as much space as non-infected control cells (Fig. 2a). When the widest distances between the cell membranes and the substratum were measured beneath the nuclei for 20 single cells, they were 0.42 ± 0.09, 0.22 ± 0.09 and <0.1 μm respectively for the mock-infected control, samples taken at 8 h p.i. and 24 h p.i. On
Fig. 6. An in situ thin section at 24 h p.i. Interspace between the cell membrane and the substratum (S) is completely lost. Arrowheads indicate virions.
In situ analysis of HSV-infected cells

Fig. 7. (a, b) EMARG of whole cultured cells at 8 h p.i. Grains are located predominantly on the nucleus (N) after labelling with \(^3\)H-thymidine for 5 to 8 h p.i.

the surface of the cells at 8 h p.i., many particles of 80 to 200 nm in diam. could be observed by SEM (Fig. 3), as has been reported from analysis of thin sections (Siminoff & Menefee, 1966; Watson et al., 1964). This situation could be better observed by in situ sectioning (Fig. 1). In Fig. 4 (a, b) from a small vesicle containing one to three virus particles placed adjacent to a microvillus projection on the cell surface, virus particles egressed via the central part of the projection or from between two projections. Fig. 4(c) shows a large vesicle which contains many virus particles, complete and incomplete, and also shows virus particles already released outside the cell. Both inside the vesicle and outside the cell, filamentous structures are observed.

At 24 h p.i., the number of virus particles at the cell surface increased and they were either single particles or in an aggregated state (Fig. 5 and 6), although the size distribution of spherical particles became broader than at 8 h p.i. (Fig. 3) and the space between the cell membrane and the substratum was completely lost by this time (Fig. 6). Margination of chromatin, duplication of the nuclear membrane, presence of virus particles predominantly at the cell surface and an increase in the number of cell surface projections were also marked. Also at this time, virus particles did not appear at the cell surface facing the substratum. In Fig. 5 (b), aggregated virions could be seen together with filamentous structures as shown in
Fig. 8. EMARG of whole cultured cells at 8 h p.i. In the case of a labelling period of 4 to 8 h p.i., many grains are located on the periphery of the cells as well as on the nuclei. (a) Peripheral part of the cell and the nucleus (N) are both covered by many grains. The inset is a twofold magnification of the area pointed by the arrowhead. (b) Peripheral parts of two cells covered with grains.

Fig. 4(c). To ascertain whether these virus particles were progeny and not infecting viruses, EMARG of whole cultured cells was performed. Fig. 7 and 8 are autoradiograms from experiments in which the cells were labelled with $^3$H-thymidine (20 μCi/ml) from 5 to 8 h p.i. and 4 to 8 h p.i. respectively. As shown in Fig. 7, where the cells were labelled for 3 h (5 to 8 h p.i.), only a small number of the silver grains could be seen on the periphery of the pseudopodium, although many virus particles were already seen at the marginal part and many grains appeared in the nucleus. When the cells were labelled for 4 h (4 to 8 h p.i.) (Fig. 8), virus particles as well as many silver grains were observed at the periphery of pseudopodia. Fig. 9 and 10 are autoradiograms of 'pulse-chase' labelling experiments. When the cells were labelled with $^3$H-thymidine for 20 min from 4.5 h p.i. and then 'chased', silver grains were present in the peripheral parts of the cell at 8 h p.i. (Fig. 10) but not at 6 h p.i. (Fig. 9). To analyse the distribution of silver grains in the cells, their numbers were counted within a regular square of 25 μm$^2$ on the nucleus, on the periphery of the cytoplasm and on the perinuclear region (Table 1). It is evident that silver grains started to appear at the peripheral parts of the cells at 7 h p.i., and at 9 h p.i. their number on the periphery of the cells increased with a concomitant decrease on the nucleus. Until 8 h p.i., the distribution of silver
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Fig. 9. EMARG of HSV-infected CV-1 cells at 6 h p.i., pulse-labelled by \(^{3}H\)-thymidine. HSV-infected CV-1 cells were labelled with \(^{3}H\)-thymidine (20 \(\mu\)Ci/ml) for 20 min at 4-5 h p.i., as described in Methods and processed for EMARG at 6 h p.i. Silver grains are located only on the nuclei (N) of the cells.

Fig. 10. EMARG of HSV-infected CV-1 cells at 8 h p.i., pulse-labelled with \(^{3}H\)-thymidine for 20 min at 4-5 h p.i. At 8 h p.i., HSV-infected and pulse-labelled CV-1 cells were processed as described. Silver grains are located not only on the nuclei (N) but also on the peripheral parts of the cytoplasm.
Table 1. Distribution of silver grains on the cells infected by HSV and pulse-labelled with $^{3}H$-thymidine*

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>Nucleus</th>
<th>Perinuclear cytoplasm</th>
<th>Periphery of the cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>$43 \pm 16^{\dagger}$</td>
<td>NS$^\dagger$</td>
<td>NS</td>
</tr>
<tr>
<td>7</td>
<td>$43 \pm 12$</td>
<td>$4 \pm 2$</td>
<td>$(17 \pm 6)^{\S}$</td>
</tr>
<tr>
<td>8</td>
<td>$32 \pm 8$</td>
<td>$5 \pm 2$</td>
<td>$16 \pm 7$</td>
</tr>
<tr>
<td>9</td>
<td>$24 \pm 9$</td>
<td>$4 \pm 2$</td>
<td>$20 \pm 7$</td>
</tr>
</tbody>
</table>

* Cells were labelled and processed as described. Twenty cells for each experimental group except one were examined for the number of silver grains within a regular square of $25 \mu m^2$ at the different sites on the cell surface.

$^{\dagger}$ Mean ± standard deviation.

$^\dagger$ NS, Not significant when compared with background.

$^{\S}$ Number in parentheses indicates mean of five cells. Only five cells out of 300 showed a significant number of grains on the periphery of the cells.

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grains at the periphery of the cytoplasm was almost restricted to the zone within $5 \mu m$ of the border of the cell. But at 9 h p.i. silver grains on the periphery of the cell started to exhibit twice as wide a distribution as at 8 h p.i., so the absolute number of silver grains on this area increased at 9 h p.i. in spite of the constancy of the density as shown in Table 1. The small numbers of silver grains halfway between the nuclei and the periphery of the cells at any time point indicate a very quick transit of mature virions from nuclei to the periphery of the cytoplasm. These observations support the idea that virus particles, which were seen at the periphery of pseudopodia at 8 h, were progeny particles and that it took at most 1 to 2 h for virions to move from the nucleus to the cell surface after maturation, as discussed below.

DISCUSSION

The experimental procedures employed in this paper enabled us to observe infected or non-infected cells in tissue culture, in situ. So far, except in the case of scanning electron microscopy, electron microscopical studies have been done using cells which were artificially treated, such as by trypsinization or scraping off, hence it appears that the real status of the cultured cells may not be assessed properly after these procedures. In our present studies, besides SEM, transmission electron microscopy of whole cultured cells (EMARG) and of thin sections (TS) in situ was performed to study transit and release of HSV particles in and from CV-1 cells. By EMARG (Fig. 7, 8, 9, 10 and Table 1) we could deduce that mature virus particles moved to the periphery of the cells within 1 to 2 h at most, taking into account that it takes about 2 h for the maturation of HSV in the nucleus (Roizman et al., 1963; Olshevsky et al., 1967). It took 3 to 4 h for the progeny virus, freshly labelled with $^{3}H$-thymidine, to reach the surface of the cells after DNA synthesis. It could be argued that those silver grains were not necessarily associated with the virion and that labelled cellular or virus DNA leaked out from the injured nuclear membrane. However, this is unlikely because, in cells labelled for 5 to 8 h p.i., distribution of the grains was almost restricted to the nuclei, although the damage to the nuclear membrane should be the same as in the case of a labelling period of 4 to 8 h p.i. A quantitative study of the distribution of silver grains was also performed in the case of pulse-labelling experiments (Table 1). Because of the difference in thicknesses inside any one cell, we could only give approximate values. The data support the interpretations mentioned above and indicate a very quick transit of virions from the nucleus to the periphery of the cells. Also, it is suggested from Fig. 1 and 6, that progeny virus particles were situated on that part of the cytoplasmic membrane where membrane movement seemed free. This part of the membrane included the dorsal part of the cells and pseudopodia and not the membrane facing the substratum. This fact suggested the idea that the virus particles moved to the cell surface with the streaming of the cytoplasm, if the flow of the cytoplasm was minimized at the fixed
part of the cell membrane. This idea can also be supported by the fact that, in cells treated with cytochalasin B, an inhibitor of cell movement by microfilaments, HSV particles were retained in the nucleus (Marciano-Cabral et al., 1977), possibly because of the inhibition of nucleoplasmic movement. At the later stages of infection, the cell membrane facing the substratum became flat and the interspace between the membrane and the substratum was almost lost (Fig. 6); this might be interpreted as the result of destruction of the cytoskeleton during the infection process (Ebina et al., 1978), since there was no sign of an increase in the number of adhesion plaques where a concentration of microfilaments should be observed (Abercrombie et al., 1971; Brunk et al., 1971). This might also be the result of cell fusion induced by the virus. At 8 h.p.i., egression of the virus particles from the cell surface became evident and the presence of filamentous structures inside or outside the cell was observed (Fig. 4c and 5b), but the nature of these structures remains to be studied. The egression of the virus from the cell surface was reconfirmed as a reverse phagocytosis process (Morgan et al., 1959; Nii et al., 1968) by in situ thin section procedures (Fig. 4 and 5b). Although the authors admit that it can be argued that phagocytosis of already released virions is taking place, virus release by reverse phagocytosis seems probable, because the number of the sites showing phagocytosis or reverse phagocytosis did not increase from 8 h.p.i. to 24 h.p.i. and was irrelevant to the increase in the number of virions on the cell surface. The site of virus release was very close to the position of the microvilli, as is the case for vesicular stomatitis virus (Holmes, 1975), or was in the midst of a microvillus projection (Fig. 4). At 8 h (Fig. 1), virus particles were predominantly in the nucleus, but at 24 h, almost all of them had moved to the cell surface (Fig. 6). This means that, under the experimental conditions used, virus replication probably ceased before 24 h p.i. Characteristic asymmetry of the site of virus release was also reported by Boulan & Sabatini (1978) in the case of MDCK cells infected by influenza virus.

Thus, we consider that the methods used here minimize artificial effects of harvesting the cells and enable us to study the true in situ situation of the infected cells.

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REFERENCES


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