Differences in the Morphology of Herpes Simplex Virus Infected Cells: I. Comparative Scanning and Transmission Electron Microscopic Studies on HSV-1 Infected HEp-2 and Chick Embryo Fibroblast Cells

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(Accepted 23 January 1979)

SUMMARY

Infection with herpes simplex virus type 1 (HSV-1) induces different morphological changes in different cell lines. This is demonstrated by comparative scanning (SEM and transmission (TEM) electron microscopic investigations of cell cultures prepared under identical conditions. SEM of HSV-1 infected HEp-2 cells reveals a slightly altered cell surface: only the number of the microvilli is reduced. Large amounts of released virions are detectable adhering to the outer plasma membrane. Ultra-thin sections show typical virus maturation steps in the nuclei (formation of nucleocapsids and virus budding from the inner lamella of the nuclear membrane) and in the cytoplasm (egress of enveloped nucleocapsids through membranous structures). HSV-infected primary chick embryo fibroblast (CEF) cells are characterized by crumpled and rough surfaces without virus particles adhering to the membrane. Ultra-thin sections exhibit atypical virus maturation with many unenveloped nucleocapsids within the cytoplasm. The distribution of HSV-induced antigen(s) on the surface of the infected cells is identical in the two cell systems as determined by the peroxidase labelling technique. The c.p.e. (as seen by phase contrast light microscopy) is similar in both HEp-2 and CEF cells: both fusion and rounding up is induced in the infected cells.

INTRODUCTION

The membranes of cells infected with herpesviruses undergo distinct morphological, biochemical and immunological changes during virus replication. The c.p.e. of HSV-1 and the altered ‘social behaviour’ of several cell lines after infections have been extensively investigated for different strains of herpesviruses (Gray et al. 1958; McNair Scott & McLeod, 1959; Nii, 1961; Nii & Kamahora, 1961b; Schneweis, 1962; Munk & Donner, 1963; Ejercito et al. 1968; Batra et al. 1978).

Many data are available on HEp-2 or HeLa cells (e.g. Roizman, 1961; Epstein, 1962; Schwartz & Roizman, 1969a, b; Heine et al. 1972), but only a few reports can be obtained concerning HSV-infected fibroblasts (e.g. Lowry et al. 1971a, b; Rodríguez & Dubois-Dalcq, 1978).

Though many electron microscopic studies exist dealing with the maturation mechanisms of the herpesvirus (Morgan et al. 1954, 1959; Falke et al. 1959; Epstein, 1962; Siegert & Falke, 1966; Nii et al. 1968a), only little information is available about the surface of infected cultured cells studied by scanning electron microscopy (e.g. Glaser et al. 1977; Fonte & Porter, 1974).
Several authors have investigated the role of the infected cell in the virus-induced c.p.e. in different cell lines (Gray et al. 1958; Hoggan & Roizman, 1959; Nii & Kamahora, 1961 a). The role of membrane alterations has been studied with respect to the contiguity of host and virus proteins (Heine et al. 1972; Heine & Roizman, 1973), to antigenic modification (e.g. Nii et al. 1968 b; Shimizu, 1971; Costa et al. 1978), to fusion-inducing factors (e.g. Falke, 1972; Levitan & Blough, 1976) and to morphological alterations (e.g. Smith & de Harven, 1973; Rodriguez & Dubois-Dalcq, 1978).

As recently reported, HSV-1 induces in HEp-2 and six other cell lines (FL, KB, HeLa, BHK-21, primary rabbit kidney, Chang-liver) an increased stability of the cellular membrane in comparison to uninfected cells (Schlehofer et al. 1979). This effect is characterized by a decreased permeability for $^{51}$Cr, by an increased resistance to Triton X-100, and, at least for HEp-2 cells, by protection from complement-mediated cytolysis after treatment with anti-HSV-1 sera. Opposite results were found in HSV-1 infected CEF cells: an enhanced permeability for $^{51}$Cr, an increased sensitivity to Triton X-100 and cytolysis after exposure to anti-HSV-1 antibodies and complement. In order to explore whether these functional events are connected with structural differences, electron microscopic techniques were applied to HSV-infected cells.

**METHODS**

**Cell cultures and media.** HEp-2 and primary CEF cells were cultivated on $8 \times 50$ mm glass coverslips in $50$ mm Petri dishes. Growth medium consisted of Eagle's minimal essential medium (MEM; Gibco, Paisley, Scotland), supplemented with $1.7$ g/l bicarbonate, $5\%$ (HEp-2) or $10\%$ (CEF) newborn calf serum (Flow, Bonn, Germany), heat-inactivated for $30$ min at $56^\circ$C and antibiotics (penicillin G: $100$ units/ml, streptomycin sulphate: $100$ $\mu$g/ml, nystatin $25$ units/ml).

**Virus.** A strain of herpes simplex virus type 1 (HSV-1) isolated in our laboratory was propagated in HEp-2 cells and assayed by plaque titration in CEF cells. In all experiments the m.o.i. was $3$ p.f.u./cell. Similar results were obtained with strains HFEM and THEA.

**Preparation of cells for electron microscopy.** Cells, grown on coverslips ($5 \times 10^5$ cells) were inoculated with HSV-1 in Eagle's MEM without serum or mock-infected and incubated at $37^\circ$C with $5\%$ CO$_2$ for $1$ h. The cells were then washed with growth medium ($37^\circ$C) and the coverslips put into glass tubes containing growth medium ($37^\circ$C) and incubated at $37^\circ$C, ensuring a horizontal position. $24$ h.p.i., if not otherwise indicated, the tubes containing the coverslips were carefully set in an upright position and the growth medium was substituted with imidazole buffer ($10$ mM-imidazole, $150$ mM-NaCl, $0.75$ mM-CaCl$_2$, $2$ mM-MgCl$_2$, HCl, pH 7.2) at $37^\circ$C. To avoid turbulence, substitution was done by constructing a gradient with an LKB Multiperpex rotational pump (Uppsala, Sweden; pumping speed: $1$ ml/min): the buffer was added through silicone rubber tubing (inner diam. $1.3$ mm, LKB) slightly under the meniscus of the growth medium, which was removed simultaneously from the bottom of the tube (gradient procedure). After the complete substitution of the medium by buffer, the first fixation medium (formaldehyde $4\%$ (w/v) and glutaraldehyde $0.25\%$ (v/v) in $0.15$ M-cacodylate HCl, pH 7.2, $5$ mM-CaCl$_2$; Mannweiler & Rutter, 1975; Karnovsky, 1965) was applied at $37^\circ$C by inverse pumping, i.e. the fixative was applied at the bottom of the tube and the buffer removed from the top. By this procedure a continuous exchange is achieved (exchange procedure). During fixation ($15$ min) the cells were allowed to attain room temperature. All the following preparative steps were carried out at room temperature. The aldehyde fixative was then substituted with imidazole buffer by the gradient procedure. Then the coverslips (still immersed in buffer) were transferred into special dishes (K. Mannweiler, personal communication) for immunological labelling by the immunoperoxidase technique (Graham & Karnovsky, 1966; Avrameas, 1969; Bretton
et al. 1972; Dougherty et al. 1972; Mannweiler & Rutter, 1973, 1974, 1976; Sternberger, 1977). The coverslips were overlaid for 5 min with anti-HSV-1 serum from rabbits, neutralizing titre 1:160, in a final dilution of 1:10. The immunizing strain was MacIntyre, VR3 (DAKO, Copenhagen, Denmark). After gentle but extensive washing with imidazole buffer, the coverslips were overlaid for 5 min with peroxidase-conjugated anti-rabbit serum IgG (final dilution 1/10, DAKO) and washed again. The cells were fixed by overlaying with excess of 1·75% (v/v) glutaraldehyde (in 0·1 M-cacodylate-HCl, pH 7·2) for 20 min. After washing with imidazole buffer the peroxidase was allowed to react by overlaying the cells with 50 mg% (w/v) 3,3’-diaminobenzidine-tetrahydrochloride (DAB, Fluka, Buchs, Switzerland) and H2O2 (0·01%, v/v) in 0·05 M-tris-HCl, pH 7·6, for 20 min. For controls, imidazole buffer was used instead of either anti-HSV serum, peroxidase-labelled antiserum or DAB reagent.

In order to prepare several coverslips under identical conditions the following procedures were carried out with the exchange apparatus described by Peters & Rutter (1974). The coverslips were carefully transferred (immersed in imidazole buffer) into the exchange apparatus container which can hold up to eight coverslips. The cells were fixed with OsO4 (1%, w/v, in 0·1 M-cacodylate, 35 mM-NaCl, HCl, pH 7·2) for 45 min, washed again with imidazole buffer and post-fixed with tannic acid (Merck, Darmstadt, Germany, 1% w/v, in 0·1 M-cacodylate, 35 mM-NaCl, HCl, pH 7·2; Simionescu & Simionescu, 1976a, b). After a final washing with imidazole buffer the cells were dehydrated by a continuous ethanol gradient. At an ethanol concentration of 100% the coverslips were removed from the container (still immersed in ethanol) and broken into two pieces. One piece of the coverslip was put back in the exchange apparatus and the ethanol exchanged with Freon 113 (Hoechst, Frankfurt, Germany). The cells were critical-point dried from Freon 13 (Hoechst, cf. Cohen et al. 1968) in a Polaron critical-point drying apparatus (Watford, England). One part of the dried coverslip was used for stereo surface replica investigations (not shown in this communication) and prepared according to the method described by Peters (1977). The other part of the critical-point dried coverslip was sputtered with gold in a Hummer sputter coater (Technics, Alexandria, Virginia, U.S.A.) in air, without cooling, at a thickness of 20 to 40 nm for SEM investigation. The second piece of the coverslip, still in 100% ethanol (see above), was flat embedded (K. Mannweiler, personal communication) in EPON 812 (Serva, Heidelberg, Germany). Ultra-thin sections, perpendicular to the coverslip, were made with a diamond knife on a Reichert OmU 3 Ultramikrotome (Reichert, Bielefeld, Germany) and contrasted with uranyl acetate and lead citrate. SEM micrographs were taken with a Philips PSEM 500 (Philips, Eindhoven, Netherlands) with a pointed tungsten filament at 12 kV and 6 nm spot size at a tilt angle of 46°. TEM micrographs were taken with a Philips EM 301 equipped with a specimen-cooling device at 60 kV for ultra-thin sections and at 100 kV for replicas.

RESULTS

As shown in SEM micrographs at low magnification, infection (24 h) of HEp-2 cells with HSV-1 leads to rounding up and fusion of relatively well conserved cells (Fig. 1). Higher magnification demonstrates large amounts of membrane-adhering released virus particles on the cell surface of rounded (Fig. 2 a), giant (Fig. 2 b) and mitotic cells (Fig. 2 c). As early as 8 h.p.i. newly synthesized HSV particles can be detected on the surface of HEp-2 cells (Fig. 3). Due to the low input m.o.i. resulting in one-step growth conditions, the detectable viruses are newly synthesized. The alteration of the cellular membrane of the infected cells is characterized only by a reduced number of microvilli in comparison to uninfected cells (Fig. 4). TEM micrographs (ultra-thin sections of flat embedded cells) show the well known steps of HSV-replication in culture cells (Fig. 5). The assembly of capsids in the nucleo-
Fig. 1. C.p.e. of HSV-1 in HEp-2 cells: rounded and fused cells, 24 h p.i., SEM.

Fig. 2. Released herpes particles on the surface of HEp-2 cells, 24 h p.i., SEM: (a) rounded cell; (b) giant cell; (c) cell in telophase.

Fig. 3. Released herpes particles on the surface of HEp-2 cells, 8 h p.i., SEM.

Fig. 4. Surface of mock-infected HEp-2 cells, SEM.
Fig. 5. Survey of HSV-maturation, HEp-2 cell, 24 h p.i., TEM.

Fig. 6. Maturation of HSV: envelopment by budding from an invagination of the inner lamella of the nuclear membrane, HEp-2 cell, 24 h p.i., TEM.

Fig. 7. Egression of enveloped virus particles through cytoplasmic channels, HEp-2 cell, 24 h p.i., TEM.

Fig. 8. Virus-induced thickening of limited areas of the plasma membrane (arrows), HEp-2 cell, 24 h p.i., TEM.
Fig. 9. C.p.e. of HSV-1 in CEF cells: rounded and fused cells, 24 h p.i., SEM.

Fig. 10. Strongly altered surface of a HSV-infected CEF cell, 24 h p.i., no virus particles adhering to the membrane, SEM.

Fig. 11. Surface of a mock-infected CEF cell, SEM.

Fig. 12. Disintegrated nucleus, unenveloped capsids and tubular structures in the cytoplasm of a HSV-infected CEF cell, 24 h p.i., TEM.

Fig. 13. Intensively disintegrated cytoplasm but a less disintegrated nucleus in a CEF cell (adjacent to that of Fig. 12), 24 h p.i., TEM.

Fig. 14. Envelopment of herpesvirus nucleocapsids in CEF cells by budding (arrow) from cytoplasmic membranous structures, TEM.
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plasm is accompanied by thickening of nuclear membrane areas (Schwartz & Roizman, 1969b), by invaginations and by condensation of chromatin near the nuclear membrane. The micrographs also demonstrate the envelopment of the capsids by budding from the inner nuclear membrane (Ben-Porat & Kaplan, 1972; Haines & Baerwald, 1976) and their egression from the perinuclear cisterna through the channels between the cytoplasmic membrane to the exterior of the cell (Darlington & Moss, 1969; Fig. 6, 7). Released particles adhere to the outer lamella of the plasma membrane which is thickened in some areas as described by others (e.g. Nii et al. 1968a; Fig. 8).

In contrast to these findings with HEp-2 cells, CEF cells show severe alterations of the surface after infection with HSV-1 as seen with the SEM (Fig. 9, cf. Fig. 1). The membrane is crumpled and rough, in contrast to the surface of uninfected cells (Fig. 10, 11). Probably due to the severe alteration of the membrane, viruses can rarely be detected adhering to the surface (Fig. 10, 12, 13). Because of the fact that only limited areas of the membrane are visible in thin sections, TEM cannot demonstrate this severe surface alteration. But TEM reveals a completely different maturation of virus as compared to HEp-2 cells: The nucleus is disintegrated in most cells early in the reproduction cycle (Fig. 12) representing a feature similar to metaphase chromosome structures. A high number of unenveloped nucleocapsids and tubular structures (Couch & Nahmias, 1969; Oda & Mori, 1976) can be seen within the cytoplasm. The nucleocapsids acquire their envelope by budding from cytoplasmic membrane structures (Fig. 14, cf. Epstein, 1962; Darlington & Moss, 1968; Fong & Hsiung, 1972). The typical envelopment and maturation of the virus in the nucleus as described for HEp-2 cells can be observed only in a few cells (Fig. 13). The distribution of virus-induced antigens is equal in the two cell lines (Fig. 15a, b, 16a, b), as shown by comparative preparation of both infected HEp-2 and CEF cells labelled with peroxidase-labelled antibodies.

Fig. 15. Herpes-induced antigens visualized with peroxidase-labelled antibodies on the surface of HEp-2 (a) and CEF cells (b), 24 h p.i., TEM.

Fig. 16. SEM micrographs of the same preparation (peroxidase-labelling) as in Fig. 15: (a) HEp-2 cell (insert: unlabelled surface at the same magnification); (b) CEF cell.
against HSV-1 antigens. This demonstrates that at least the distribution of immunoreactive proteins is not responsible for the morphological differences of the membrane. Non-specific reactions in both infected and uninfected cells were excluded by control experiments with buffer instead of either anti-HSV-serum, peroxidase-labelled antiserum, or DAB-reagent. Furthermore, the c.p.e. (as seen with phase contrast light microscopy) is similar for both HEp-2 and CEF cells: rounding up and fusion of infected cells. The observed differences do not influence the yield of infectious HSV-1 particles and the kinetics of virus reproduction are the same for both HEp-2 and CEF cells (Schlehofer et al. 1979).

**DISCUSSION**

In this study we have presented evidence that the cell involved in HSV infection plays a definitive role in determining (i) the electron microscope-visible features of c.p.e., (ii) the membrane changes that occur during virus reproduction and (iii) the mechanism of virus maturation. The morphological surface alterations of different cell lines can be completely different even when the virus antigens in the surface membrane are equally distributed. In order to visualize these morphological differences it is necessary to apply valid comparative techniques and to prepare cells for electron microscopy carefully (Peters & Rutter, 1974; Gusnard & Kirschner, 1977) conserving distinct differences and preserving virus particles on the cell surface. SEM reveals new aspects of infected cells because it provides information about the whole cell surface and about the cells in their 'social behaviour' (Ejercito et al. 1968). Consequently, membrane alterations and c.p.e. can be observed under optical conditions different from TEM (ultra-thin sections, surface replicas, freeze fracture) overcoming the gap between light and transmission electron microscopy. Nevertheless, TEM (ultra-thin sections) of identically prepared cultures is necessary to elucidate the changes of the interior structures of infected cells. The stronger c.p.e. and the stronger membrane alterations in CEF compared to HEp-2 cells after infection with HSV-1 corresponds to our findings of a more fragile cell membrane in infected CEF contrasting with the stabilized membrane of HEp-2 cells (Schlehofer et al. 1979). This could explain the fact that large numbers of virions adhere to the surface of HEp-2 cells, whereas the membrane of CEF cells is not able to retain virus particles. The different mechanism of virus-maturation in the two cell lines demonstrates the essential influence of cellular factors on the reproduction of HSV resulting even in different physical properties (buoyant density, CsCl) of the mature virions (Gray et al. 1958; Hoggan & Roizman, 1959; McNair Scott & McLeod, 1959; Nii, 1961; Nii & Kamahora, 1961a; Spear & Roizman, 1967).

The authors acknowledge the excellent technical assistance of Miss L. Macke and Miss A. Brehmer (virus and cell preparations) and of Miss A. Hennig (electron microscopy).

**REFERENCES**


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*(Received 24 November 1978)*