Mouse Elberfeld (ME) Virus Determines the Cell Surface Alterations when Mixedly Infecting Poliovirus-infected Cells

By HEINZ ZEICHHARDT,* JÖRG R. SCHLEHOFER,† KLAUS WETZ, HARTMUT HAMPL‡ AND KARL-OTTO HABERMEHL

Institut für Klinische und Experimentelle Virologie, Freie Universität Berlin, Hindenburgdamm 27, D-1000 Berlin 45, Federal Republic of Germany

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SUMMARY

The surface alterations of HEp-2 cells induced by mixed infection with two different picornaviruses (poliovirus and ME virus) were compared by scanning electron microscopic and transmission electron microscopic studies and by 51Cr-release assay. The contribution of each of the viruses to the resulting surface changes was discernible, as investigations on the chronology of the cytopathic alterations demonstrated that the changes were distinct for either virus. The surface of ME virus-infected cells was characterized by large membranous structures ('sheets' and blebs) representing huge vacuoles. These sheets were not seen in poliovirus-infected cells. Poliovirus induced more prominent cell pycnosis, elongation of filopodia and condensation of collapsed microvilli on the cell surface than ME virus. Mixed infection with these two viruses led to surface alterations typical for ME virus. These ME virus-specific changes occurred irrespective of poliovirus reproduction or its inhibition by guanidine. ME virus-specific alterations also predominated in cytolytic membrane damage as expressed by 51Cr-release from infected cells. 51Cr-release was more pronounced from ME virus than from poliovirus-infected cells, even when ME virus reproduction was suppressed by interfering poliovirus. However, alteration of the internal structures of the infected cells was only dominated by ME virus when the reproduction of poliovirus was suppressed.

INTRODUCTION

Scanning electron microscopic (SEM) and transmission electron microscopic (TEM) ultrastructural investigations on picornavirus-induced cytopathic changes have only been carried out with cells infected with one type of virus at a time. TEM: poliovirus and coxsackievirus (Stuart & Fogh, 1961; Dales et al., 1965; Mattern & Daniel, 1965; Bienz et al., 1973, 1980; Lenk & Penman, 1979); encephalomyocarditis (EMC) and mengovirus (Dales & Franklin, 1962; Amako & Dales, 1967); foot-and-mouth disease (FMD) virus (Yilma et al., 1978); Theiler's murine encephalomyelitis (TME) virus (Friedmann & Lipton, 1980). SEM: FMD virus (Yilma et al., 1978); porcine enterovirus (Sulochana & Derbyshire, 1977); echo, coxsackie and poliovirus (Yoshii et al., 1976). These reports showed that different viruses induce specific membrane alterations in the interior of the cell and/or on its surface. Dual infection of cells with two different picornaviruses has only been studied using

* Present address: Institut für Virologie, Zentrum für Hygiene der Universität Freiburg, Hermann-Herder-Str. 11, D-7800 Freiburg i.Br., Federal Republic of Germany.
† Present address: Department of Microbiology, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232, U.S.A.
functional criteria (McCormick & Penman, 1968; Shirman et al., 1973; Diefenthal et al., 1973; for detailed references, see Zeichhardt et al., 1981).

We have recently shown that virus-induced changes of cellular membranes can occur independently of viral reproduction in dual infections (Zeichhardt et al., 1981). Poliovirus, even when inhibited by guanidine, induces a cytoplasmic membrane complex that can act as a site for the replication of mixedly infecting ME virus. Furthermore, ME virus reproduction is accelerated due to exploitation and modification of the poliovirus-induced complex.

In this paper we describe the ultrastructural changes of cells doubly infected with poliovirus and ME virus under conditions in which the replication of one or the other virus was inhibited. Under all conditions of mixed infection the surface alterations were dominated by ME virus-specific changes while the internal alterations were mainly influenced by poliovirus, provided that poliovirus reproduction was not suppressed by guanidine.

METHODS

Cells. As previously described (Schlehofer et al., 1979b), HEp-2 cells were cultivated on coverslips (8 mm × 50 mm) in Petri dishes in Eagle's minimal essential medium (MEM, Gibco) supplemented with bicarbonate (1.7 g/l), 5% heat-inactivated newborn calf serum (Flow Laboratories) and antibiotics. In most experiments the growth medium was supplemented with 2.6 mM-guanidine-HCl (Schwarz/Mann, Orangeburg, N.Y., U.S.A.).

Viruses. Poliovirus type 1 (strain Mahoney) and ME (Mouse Elberfeld) virus (laboratory strain) were used throughout the studies. The viruses were propagated and titrated on HEp-2 cells. Plaque titration experiments with poliovirus and ME virus were performed in the presence of 0.01% DEAE-dextran and/or 2.6 mM-guanidine (see Zeichhardt et al., 1981).

Conditions of infection. The methods for single and superinfection with poliovirus and ME virus have been previously described (Zeichhardt et al., 1981). Experiments were carried out (in the presence of 2.6 mM-guanidine) either as a single infection with poliovirus or ME virus, or as a simultaneous infection with both viruses or as a superinfection (preinfection with poliovirus 4 h prior to superinfection with ME virus). Cells with single or simultaneous double-infection were also examined in the absence of guanidine. In all experiments the m.o.i. was 20 for each of the viruses. All media were prewarmed (37 °C) overnight in an atmosphere of 5% CO₂.

Preparation of specimens for electron microscopy. After inoculation with the respective virus(es) for 30 min the cells were washed three times with growth medium (37 °C) and the coverslips were transferred into plastic test tubes containing growth medium (37 °C). The cells were incubated (5% CO₂ at 37 °C) ensuring a horizontal position of the coverslip. At various times after infection (3, 4-5, 6, 8, 10 and 12 h post-infection) the cells were carefully fixed (formaldehyde and glutaraldehyde at 37 °C, osmium tetroxide and tannic acid at 22 °C), demineralized and dehydrated by gradients (see Rostgaard & Tranum-Jensen, 1980) and prepared for SEM and TEM by the previously described exchange method (see Schlehofer et al., 1979b). In addition, to exclude dehydration artefacts, cell cultures were freeze-dried (−90 °C) immediately after the aldehyde fixation.

For the analysis of early stages of infection (0-5 or 1 h post-infection) coverslips were transferred 8 h prior to infection from the Petri dishes into plastic tubes (for recovery of the cells) where they were infected as follows: with the pumping device used for the exchange method, the relevant inoculum was substituted for the growth medium, avoiding turbulence. The cells were washed and fixed by the same device. These precautions to minimize mechanical cell damage were unnecessary for later stages of infection, when the cells were allowed to recover for more than 1 h.

The procedures described allowed comparative SEM and TEM analysis of cells from the same coverslip and ensured identical preparation of cells from differently infected cultures.
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(for details, see Peters & Rutter, 1974; Baigent et al., 1978; Peters, 1980). For SEM, critical-point-dried or freeze-dried cells were sputter-coated with Au-Pd on a cooled stage in an argon atmosphere in a Hummer II (Technics, Alexandria, Va., U.S.A.) at a thickness of 20 to 30 nm. For TEM, cells flat-embedded in low viscosity epoxy resin (Spurr, 1969) were sectioned perpendicular to the coverslip with a diamond knife on a Reichert Ultracut microtome (Reichert, Bielefeld, F.R.G.) and contrasted with uranyl acetate and lead citrate. Micrographs were taken with Philips electron microscopes (EM 301 and PSEM 500).

51Cr-release assay. Cells, labelled by incubation in growth medium containing 51Cr (Na2[51Cr]O4, Amersham, Braunschweig, F.R.G., 10 µCi/ml) for 1 h prior to infection, were inoculated with the respective virus (for details, see Schlehofer et al., 1979a). Duplicate samples of the supernatant (25 µl) were taken at intervals and counted for radioactivity in a Packard gamma scintillation counter. The ct/min/ml at a given time after infection were calculated with the following formula whereby preceding samples are taken into account:

\[
\text{ct/min}_{(t)}/\text{ml} = \left( \frac{s_{(t)} \times V_i}{V_s} + s_n - s_{(t)} \right) \times \frac{1}{V_i}
\]

where ct/min_{(t)}/ml = actual ct/min in the supernatant at a given time (per ml); s_{(t)} = ct/min of a sample taken at a given time; V_i = initial volume of the supernatant (in ml); V_s = volume of sample (in ml); s_n = sum of the ct/min of all samples so far taken [including s_{(t)}].

RESULTS

Mixed-infections with poliovirus and ME virus were carried out under conditions in which the replication of one of the viruses was suppressed. In order to determine the contribution of each of the viruses to the cytopathic changes induced in these mixed infections, the ultrastructural changes of cells infected with either virus were analysed by SEM and TEM.

ME virus

SEM micrographs revealed that ME virus induced novel structures in HEp-2 cells. In comparison to mock-infected cells (Fig. 1 a) the surfaces of infected cells were characterized by huge blebs and 'sheet-like structures' (diam. >20 µm, Fig. 1 b to f). Such surface changes could be observed as early as 4.5 h post-infection (Fig. 1 b) and increased in number later in the course of infection (for 12 h post-infection, see Fig. 1 d). From analysis of many preparations at different times after infection (4.5, 6, 8, 10, 12 h post-infection) it was concluded that the huge blebs became sheet-shaped when collapsing or rupturing (Fig. 1 e). When blebs of adjacent cells contacted each other or the surfaces of neighbouring cells, the membranes appeared to merge (compare Fig. 1 c, f). Later in infection when the cells became more pycnotic and rounded up, the sheets were stretched over long distances between the cells (often up to 80 µm, Fig. 1 c, d). Freeze-dried cell controls were examined in order to exclude the possibility that the blebs and sheets were artefacts formed during dehydration. Similar membrane structures were seen in ME virus-infected cells prepared in this way (not shown).

Comparative ultra-thin sections (TEM) of ME virus-infected cells showed that the blebs were electron translucent (Fig. 2 a) like all the vacuoles of various sizes that were characteristic for the cytoplasmic alterations induced by ME virus (Fig. 2 b). Furthermore, after infection most cellular organelles appeared to be swollen in a cytoplasm which had become electron translucent. In the nucleus most of the chromatin was condensed and marginated leaving a vacant karyoplasm (Fig. 2 b).
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Fig. 1. ME virus-induced surface changes of HEp-2 cells. (a) SEM of mock-infected HEp-2 cells at 8 h post-infection (12 kV, 46° tilt angle). (b to f) SEM of ME virus-infected HEp-2 cells: (b) at 4-5 h post-infection, two large blebs are demonstrated, one of these blebs is collapsed giving rise to sheet formation (12 kV, 46° tilt angle); (c) at 8 h post-infection, high frequency of sheet-like structures stretching over several cells, partially fused (12 kV, 46° tilt angle); (d) at 12 h post-infection, cells are shrunken and rounded up, sheet-like structures predominate (12 kV, 21° tilt angle). Bar markers in (a to d) represent 10 μm. (e) High magnification of a collapsing bleb becoming sheet-shaped, at 8 h post-infection (12 kV, 46° tilt angle); (f) high magnification of ME virus-induced sheets. The fusion areas between adjacent sheets can be distinguished. Small holes indicate the fragility and thinness of the membranes, at 8 h post-infection (12 kV, 46° tilt angle). Bar markers in (e) and (f) represent 1 μm.

Poliovirus

In SEM, blebs and sheet-like structures were not observed in poliovirus-infected cells. In comparison to ME virus-infected cells (Fig. 1b to d) the most striking features were many extremely elongated filopodia (up to 70 μm) and collapsed microvilli condensed on the surface of cells that were more rounded and pyknotic than after ME virus infection (Fig. 3a, b). As early as 3 h post-infection the elongation of filopodia could be observed on cells that were otherwise not altered (not shown). Later in infection (10 h post-infection) the filopodia of
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Fig. 2. TEM (ultra-thin sections) of ME virus-infected HEp-2 cells at 8 h post-infection. (a) Giant blebs and sheets are sectioned revealing electron translucent vacuoles. Vacuolated cytoplasm with swollen mitochondria is demonstrated. (b) Margination of chromatin in an electron translucent nucleus. Vacuolated cytoplasm, many lysosomes, disintegrated and swollen cell organelles. Bar markers in (a) and (b) represent 1 μm.
Fig. 3. Poliovirus-induced alterations. (a to c) SEM of poliovirus-infected HEp-2 cells: (a) cells are rounded up exhibiting elongated filopodia, at 8 h post-infection (12 kV, 46° tilt angle); (b) high magnification showing condensation of collapsed microvilli on the cell surface, at 8 h post-infection (12 kV, 46° tilt angle); (c) an example of elongated and merged filopodia between different cells at 10 h post-infection (12 kV, 80° tilt angle); (d) TEM (ultra-thin section) of a poliovirus-infected pycnotic cell at 8 h post-infection revealing typical condensation of chromatin arranged in patches in a lobed nucleus and clusters of vesicles in the cytoplasm. Bar markers represent 10 μm in (a, c) and 1 μm in (b, d).
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Fig. 4. Reproduction kinetics showing interference between poliovirus and ME virus; poliovirus-induced suppression of ME virus reproduction in simultaneously infected HEp-2 cells. □, Poliovirus in a single infection; ○, ME virus in a single infection; ■, progeny of poliovirus in simultaneously infected cells; ●, progeny of ME virus in simultaneously infected cells; ◐, total virus offspring in simultaneously infected cells. Differentiation between poliovirus and ME virus was performed by plaque titration on HEp-2 cells in the presence of 0.01% DEAE-dextran and/or 2.6 mM-guanidine as described by Zeichhardt et al. (1981).

Fig. 5. Simultaneous infection with poliovirus and ME virus. (a) SEM of HEp-2 cells simultaneously infected with poliovirus and ME virus at 8 h post-infection. The ME virus-induced sheets and blebs dominate the surface alterations (12 kV, 46° tilt angle). Bar marker represents 10 μm. (b) TEM (ultra-thin section) of simultaneously infected HEp-2 cells at 8 h post-infection revealing internal changes typical of poliovirus-infected cells (see Fig. 3d). Bar marker represents 1 μm.

neighbouring cells appeared to merge (Fig. 3c). Ultra-thin sections of poliovirus-infected cells confirmed the well-known nuclear and cytoplasmic changes: lobed nuclei with irregular distribution of condensed chromatin and vesicles arranged in clusters in the cytoplasm (Fig. 3d).

Simultaneous infection of poliovirus and ME virus

When both viruses infected the cells simultaneously, ME virus reproduction was suppressed by poliovirus (Fig. 4). Under these conditions, however, surface alterations (blebs and sheets) were attributable to ME virus whereas specific nuclear and cytoplasmic changes were attributable to poliovirus (Fig. 5a, b).

Single infections in the presence of guanidine

Further experiments were carried out in the presence of guanidine which had previously been shown to inhibit poliovirus but facilitate the ME virus replication in mixedly infected
Fig. 6. Single and simultaneous infections with poliovirus and ME virus in the presence of guanidine. 
(a) SEM of surface of ME virus-induced blebs in the presence of guanidine at 10 h post-infection. Small 
protuberances are discernible on the surface of the blebs (6 kV, 46° tilt angle). (b) SEM of HEp-2 cells
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Fig. 7. ME virus superinfection of poliovirus-preinfected cells in the presence of guanidine. (a) SEM of HEp-2 cells ME virus-infected 4 h after preinfection with poliovirus in the presence of guanidine (total infection time 10 h). ME virus-induced blebs occur in a frequency as expected for a 6 h single infection with ME virus (12 kV, 46° tilt angle). Bar marker represents 10 μm. (b) TEM (ultra-thin section) of a cell infected as described in (a). ME virus-specific changes in the interior of the cell are prevalent. Bar marker represents 1 μm.

cells (Zeichhardt et al., 1981). To study the influence of the drug on the cellular morphology, cells which were either mock-infected or infected with ME or poliovirus were investigated. Mock-infected, guanidine-treated and untreated cells were almost indistinguishable (not shown). However, many small protuberances were seen on some of the typical blebs in ME virus-infected cells (Fig. 6 a) whereas in the absence of guanidine such rough surfaces were rarely seen on these structures (see Fig. 1 c to f). As expected, cells infected with poliovirus in the presence of guanidine lacked the typical poliovirus-specific surface alterations, since poliovirus replication was inhibited (Fig. 6 b). Nevertheless, in the presence of guanidine the infected cultures were not as confluent as mock-infected controls: many cells had shrunk leaving free areas in between. Zeiotic blebs occurred more frequently in these polio-virus-infected cells than in controls. These minor alterations might be results of the virus-induced shut-off of host cell macromolecular synthesis (Holland, 1964; Diefenthal et al., 1971; Helentjaris & Ehrenfeld, 1977).

Mixed-infections in the presence of guanidine

Superinfection with ME virus resulted in cytopathic changes typical for ME virus irrespective of the length of preinfection with poliovirus in the presence of guanidine. This was shown by SEM and TEM for cells after simultaneous infection (Fig. 6 c to e) as well as after superinfection of poliovirus-preinfected cells (Fig. 7 a, b). As in single infections with ME virus the influence of guanidine was characterized by rough surfaces of the sheet-like structures (Fig. 6 c, d, see also Fig. 6 a).

Analysis of cytolysis of infected cells by 51Cr-release

From 10 h after infection the differences of 51Cr-release induced by the different modes of infection were clearly discernible (Fig. 8). In comparison to the kinetics of 51Cr-release from infected with poliovirus in the presence of guanidine at 10 h post-infection. In spite of inhibition of poliovirus reproduction, the cell surface is slightly altered (see Fig. 1 a) (12 kV, 46° tilt angle). (c, d) SEM of HEp-2 cells simultaneously infected with poliovirus and ME virus in the presence of guanidine at 8 h post-infection. Sheet-like structures typical of ME virus predominate. The higher magnification (d) demonstrates the protuberances on the surface of the blebs indicating the influence of guanidine (see Fig. 6 a) (12 kV, 46° tilt angle). (e) TEM (ultra-thin section) of HEp-2 cells simultaneously infected with poliovirus and ME virus in the presence of guanidine 8 h post-infection. ME virus-typical changes in the interior of the cell predominate. Bar markers represent 1 μm in (a, e) and 10 μm in (b to d).
Fig. 8. $^{51}$Cr-release from HEp-2 cells differently infected with poliovirus and/or ME virus. $^{51}$Cr-labelled cells were inoculated with the respective virus(es) at time 0. At 4 h post-infection all cell cultures were washed with growth medium in order to obtain identical conditions for the measurement of $^{51}$Cr-release in single and superinfection experiments. Data represent the average values of four experiments. ●—●, cells single-infected with ME virus; ▲—▲, cells single-infected with poliovirus; ■—■, cells simultaneously infected with poliovirus and ME virus; +—+, cells superinfected with ME virus 4 h after infection with poliovirus: ○—○, mock-infected cells; •—•, cells single-infected with ME virus in the presence of guanidine; ●—●, cells single-infected with poliovirus in the presence of guanidine; ■—■, cells simultaneously infected with poliovirus and ME virus in the presence of guanidine; +—+, cells superinfected with ME virus 4 h after infection with poliovirus in the presence of guanidine; ○—○, mock-infected cells in the presence of guanidine.

Poliovirus-infected cells, $^{51}$Cr-release from ME virus-infected cells was accelerated and increased twofold. When cells were simultaneously infected with both viruses, ME virus induced an increase of $^{51}$Cr-release although ME virus reproduction was reduced by interfering poliovirus (see Fig. 4). This effect could not be observed when cells were preinfected with poliovirus for 4 h. However, when in a simultaneous infection poliovirus reproduction was suppressed by guanidine, the ME virus-induced enhancement of $^{51}$Cr-release was advanced by 1 h compared to cells infected with ME virus only. An advance of as much as 2 h was attained when cells were preinfected with poliovirus for 4 h in the presence of guanidine. This is expressed by the steeper slope of the $^{51}$Cr-release curve. Hence, the level of $^{51}$Cr-release after 16 h single infection with ME virus was attained after only 12 h ME virus infection under these superinfection conditions. This demonstrates that poliovirus, when inhibited by guanidine, contributed to the cytolysic membrane alterations induced by the ME virus.

**Discussion**

In this paper we present evidence that in HEp-2 cells doubly infected with poliovirus and ME virus, the alterations of the cell surface were determined by ME virus. This could clearly be established because each of the viruses induced characteristic surface changes which were easily distinguishable in the doubly infected cell. The SEM investigations revealed large blebs as typical features of ME virus-infected cells which later in infection gave rise to huge sheet-like structures (see Zeichhardt et al., 1980b). These membranous structures were proven to be virus-induced since they were also seen in non-dehydrated freeze-dried cells and so were not artefacts produced during preparation (see Boyde et al., 1972). Similarly, blebs have been observed with FMD virus-infected cells (Yilma et al., 1978), but the ME virus-induced blebs were up to 10 times larger in diameter.
Poliovirus-infected cells were characterized by elongated filopodia and condensed microvilli collapsed at the cell surface. The elongation of filopodia has already been described by light microscopic studies on living cells (Diefenthal & Habermehl, 1967, 1969) as well as by SEM (Yoshii et al., 1976). Due to inadequate preparation methods, however, these SEM investigations did not provide detailed information about the cell surface.

In mixed infections with both viruses, the ME virus-induced cell membrane alterations always dominated, irrespective of suppression of ME virus reproduction by interfering poliovirus. However, TEM studies revealed that the internal changes of the doubly infected cells were determined by poliovirus unless poliovirus was inhibited by guanidine.

We have recently shown that the reproduction of ME virus is accelerated by up to 3 h when cells are preinfected with poliovirus in the presence of guanidine (Zeichhardt et al., 1981). This effect was found to be due to cytoplasmic membranes that are induced by non-replicating poliovirus and subsequently modified and exploited by ME virus as a site for ME viral RNA polymerase (Zeichhardt et al., 1980a). These changes of internal membranes (resulting in an accelerated reproduction of ME virus) are consistent with alterations of the surface membrane. The ME virus-induced cytolysis is accelerated by 2 h in cells that are preinfected with poliovirus inhibited by guanidine. The advanced enhancement of $^{51}$Cr-release in these superinfections, however, was not matched by a similarly advanced generation of the ME virus-induced sheets, showing that the large surface area of the sheets is not responsible for the pronounced $^{51}$Cr-release of ME virus-infected cells. Furthermore, the sheet-like structures are certainly not the cause for the consistently more prominent cytolytic damage of the cell membrane after infection with ME virus than after poliovirus infection. The characteristic differences in membrane permeability induced by the two viruses (see Schlehofer et al., 1979a) were only observed 10 h post-infection with either virus, whereas extensive sheet formation was detected as early as 8 h post-infection with ME virus.

Our studies on the membrane alterations in cells doubly infected with poliovirus and ME virus allowed us to investigate the cellular status under conditions when the replication of one of the viruses was suppressed. The inhibited virus may influence the changes in the cell's interior and on its surface as well as the reproduction of the non-inhibited virus.

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