Cytochemical and Electron Microscopical Observations on the Presence and Origin of Adenosine Triphosphatase-Like Activity at the Surface of Two Myxoviruses

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ABSTRACT

DAWSON, C. R. (The Middlesex Hospital Medical School, London, England), M. A. EPSTEIN, AND K. HUMMELER. Cytochemical and electron microscopical observations on the presence and origin of adenosine triphosphatase-like activity at the surface of two myxoviruses. J. Bacteriol. 89:1526-1532. 1965.—HeLa cells infected with either fowl plague virus (FPV) or Newcastle disease virus (NDV) were examined in thin sections by electron microscopy. Preparations were studied both after direct fixation and embedding and after the application of cytochemical staining for enzymes splitting adenosine triphosphate. Viral particles were identified by their size and characteristic structure, and were found to form at the cell surface by budding out through structurally altered plasmalemma. After cytochemical staining for adenosine triphosphatase activity, extracellular FPV or NDV particles lying close against cell membranes with enzyme activity likewise carried this function, whereas those particles which were associated with cell surfaces without reaction product were themselves free from it. This correspondence between enzyme function in cell membranes and the outer viral membranes of newly formed particles adjacent to them indicates that surface enzymatic capability of the host cell survives even when the cell membrane undergoes morphological and antigenic alteration into myxovirus outer membrane.

Electron-microscope studies of myxovirus-infected cells have indicated on morphological grounds that the plasmalemma is altered structurally and then utilized to form the outer coat of the newly assembled viral particles as these bud out to escape from the cell (Morgan, Rose, and Moore, 1956; Morgan et al., 1961). Such an incorporation of cell membrane to form the outer viral envelope would explain the presence in myxoviruses of lipids similar to those of the host cell (Kates et al., 1962).

All other viruses which are released from cells by budding likewise use cell membrane to form the outer covering of the infective particles; this mechanism has been found with herpesvirus (Epstein, 1962b), the fowl leucosis viruses (Heine et al., 1961, 1962a), and several murine tumor viruses (Lasfargues et al., 1959; de Harven and Friend, 1960; Dalton et al., 1961). In the case of two of these membrane-bounded agents, strain A myeloblastosis virus and herpes simplex virus, enzymatic function is known to be carried to the mature particles together with the cell membrane acquired during the budding (Novikoff et al., 1962; Epstein and Holt, 1963b). However, with myxoviruses, the cell membrane is altered strikingly by the formation of an outer layer of spikes (Morgan et al., 1956; Horne et al., 1960) and the acquisition of a specific antigen, probably viral hemagglutinin (Morgan et al., 1961; Morgan, Rifkind, and Rose, 1962). Because it is not known whether functional capabilities can survive when host-cell membrane undergoes such radical changes on becoming viral envelope, experiments were undertaken with myxovirus-infected cells to follow the fate of a cell surface enzyme during viral budding and release. For this work, cytochemical methods adapted for electron microscopy were used to study the surface adenosine triphosphatase-like activity of HeLa cells (Epstein and Holt, 1963a) during infection either with fowl plague virus (FPV) or Newcastle di-

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ease virus (NDV), these being chosen to have an example of each of the two subgroups into which myxoviruses have been divided (Waterson, 1962).

**Materials and Methods**

**Virus strains.** A strain of FPV was obtained through the kindness of H. G. Pereira; it had been adapted to tissue-culture cells by 10 passages in human embryo lung cells and 2 passages in HeLa cells. The virus was received in sealed ampoules frozen to −70 C and was stored at that temperature. NDV of the Victoria strain from L(MCN) cells (Rodriguez and Henle, 1964) was used after having been passed twice in the allantoic sac of embryos from hens’ eggs. This material was likewise stored in sealed ampoules at −70 C.

**Maintenance of HeLa cells.** Stock HeLa cells were grown in 6-oz flat medical bottles as described previously (Pereira and Kelly, 1957; Epstein and Powell, 1960). When confluent sheets of cells developed, they were either used as a source of new cultures or were infected for the experiments.

**Stock virus seed.** A HeLa cell culture showing marked cytopathic changes 4 days after infection with FPV was harvested, the cells and medium were diluted with an equal volume of a suspending fluid described elsewhere (Epstein, 1958), and 1-ml volumes of this were stored in sealed ampoules at −70 C for use as the stock virus seed. When thawed, this material had a 50% end point per milliliter of 10^4.8 as measured by cytopathic effect in HeLa cells. For NDV, infected allantoic fluid was itself used as seed without further passage. After storage at −70 C, this egg fluid had a titer of 4.1 × 10^6 plaque-forming units per milliliter when titrated on chick embryo fibroblasts.

**Infection of HeLa cells for the experiments.** For purposes of infection, the FPV seed was diluted 1:10 and the NDV seed 1:100 with the suspending fluid. Cell sheets were washed twice with Gey’s balanced salt solution, and were then covered with 2 ml of virus inoculum, and left for 1 hr at 37 C. After this the inoculum was removed, 10 ml of medium III of Pereira and Kelly (1957) were added, and the bottles were returned to the incubator.

**Collection, fixation, and cytochemical staining of cells.** Infected cell sheets were harvested when cytopathic changes were marked, about 4 to 5 days after inoculation with FPV and 14 to 24 hr after inoculation with NDV. This was done by removing the maintenance medium, replacing it with 2 ml of Gey’s solution, and, with this, washing the cells into suspension by gently pipetting. The cell suspension was drawn into a syringe and was fixed by squirting into glutaraldehyde, after which part of it was prepared directly for electron microscopy, and part was stained cytochemically for adenosine triphosphatase activity exactly as in earlier work (Epstein and Holt, 1963a), except that 1% glutaraldehyde was used; as before, control cytochemical treatments were included in each experiment.

**Preparation of cells for electron microscopy.** Suspensions of glutaraldehyde-fixed cells which were not to be stained cytochemically, and of cells which had been exposed to the cytochemical staining or control reagents, were postfixed with osmium, dehydrated, and embedded by methods already described (Epstein and Holt, 1963a).

**Microtomy and electron microscopy.** Sections were cut with glass knives on a Porter Blum microtome; were mounted on carbon-coated grids (Watson, 1965); were contrast-stained with uranyl acetate (Watson, 1958; Epstein and Holt, 1963a), lead acetate (Millonig, 1961), or a combination of both; and were examined in a Siemens Elmskop I electron microscope.

**Experimental procedure.** In four experiments, HeLa cells infected with FPV were collected at intervals during the period when cytopathic change was progressing to a maximum, and were fixed and prepared for electron microscopy either directly or after cytochemical staining for adenosine triphosphatase-like enzymes. The relationship of adenosine triphosphatase activity in the cell membrane and outer viral membrane was studied with special reference to viral release by budding.

In three further experiments, this relationship was investigated in a similar manner in HeLa cells infected with NDV.

**Results**

**General observations.** The fine structural organization of the HeLa cells infected with either FPV or NDV closely resembled that described for uninfected cells of this strain (Epstein, 1961; Bruni, Gey, and Svatelis, 1961), but it was observed that, after glutaraldehyde fixation, fewer pinocytosis vesicles and more rough cisternae were present in the peripheral cytoplasm than has been reported after osmium fixation alone. This feature has likewise been noted in another context in uninfected HeLa cells fixed with glutaraldehyde. The appearance of the infected cells after cytochemical staining for adenosine triphosphatase activity was exactly similar to that reported for both uninfected HeLa cells and HeLa cells supporting the growth of herpesvirus (Epstein and Holt, 1963b). Enzyme reaction product was never seen in any of the cytochemical control preparations.

**Site and structure of FPV.** Viral particles were not observed within the infected cells, but were numerous around them close to the plasmalemma (Fig. 1). The virus was identified by its characteristic electron-opaque outer limiting membrane surrounded by a hazy lighter zone (Fig. 2) and by its size; two forms of particle were encountered, rounded or oval ones about 80 to 90 μ in diameter, and filamentous forms of the same size across the smallest dimension but extending up to about 1.5 μ in length (Fig. 2). These viral filaments were present in profusion...
Fig. 1 and 2. Electron micrographs of thin sections cut through HeLa cells infected with FPV. Glutaraldehyde fixation, followed by osmium fixation and epoxy resin embedding were used; the sections were contrast stained with uranyl acetate and lead acetate.

Fig. 1. Survey picture of peripheral cytoplasm in a FPV-infected HeLa cell with the cell membrane crossing the center of the field. Virus particles, both spherical and filamentous, are only present outside the cell (arrows), lying close to the plasmalemma. The cytoplasm is packed with ribonucleoprotein particles and also contains some rough elements of the endoplasmic reticulum (er). X35,000.

Fig. 2. Detail of an intercellular space between two FPV-infected cells; one cell membrane lies on the extreme right of the field, and the other, thrown into numerous processes, on the left. Spherical (s) and filamentous (f) forms of the virus lie between the cell surfaces. The outer viral membrane is covered by an amorphous hazy outer zone about 10 nm in width; this zone corresponds to the surface projections seen in negative contrast preparations (Horne et al., 1980). X135,099.
(Fig. 2), their production being a known characteristic of those myxoviruses such as FPV which are similar to influenza virus (Morgan et al., 1956; Waterson, 1962).

The hazy light outer zone of the particles, irrespective of their shape, measured about 10 μm in width (Fig. 2), and corresponded to the zone of projections seen in negative-contrast preparations of myxoviruses (Horne et al., 1960). The dense limiting membrane within this zone was found to be continuous with the plasmalemma where budding was taking place and where filaments were observed while still attached to the cell surface (Fig. 2). The center of the virus in both its forms presented an empty appearance, but with some amorphous material sometimes applied to the inner face of the dense limiting membrane.

Site and structure of NDV. As with FPV, particles of NDV were only found outside the plasmalemma of infected cells. The virus was similar in structure to FPV and also occurred in two forms (Fig. 3 and 4). It was, however, larger and showed more variability in size, the rounded particles measuring about 90 to 140 μm in diameter, which corresponded to the width of the elongated forms. The latter were rod-shaped (Fig. 3) rather than filamentous, extending only to about 600 μm in length, and were considerably fewer in number than the filaments of FPV, as would be expected with a myxovirus of the NDV subgroup (Waterson, 1962).

Relation of virus to enzyme distribution. The distribution of electron-opaque enzyme reaction product at the surface of the HeLa cells in cultures infected either with FPV or NDV was very similar to that already reported for uninfected cultures (Epstein and Holt, 1963a). Although there was some variation from culture to culture, about half the infected cells in any given section were surrounded by the dense material; the normal type of enzyme distribution was thus present, irrespective of virus production, in just the same way as was found with HeLa cells infected with herpesvirus (Epstein and Holt, 1963b).

After cytochemical staining, virus structure was sufficiently well preserved (Fig. 5 to 7) by comparison with material prepared without such treatment (Fig. 1 to 4) for each of the viruses to be recognized by its shape, by its dense limiting membrane with lighter outer zone, by its relatively empty center, and by its size (Fig. 5 to 7).

Where extracellular virus was found lying close to cells with a deposit of electron-opaque material at their plasma membranes, the particles were surrounded by similar material, this correspondence being observed both with FPV (Fig. 5) and with NDV (Fig. 6 and 7). In contrast, mature virus of either type derived from, and lying close to, surface membranes without enzyme reaction product was itself invariably unstained. Where staining was present around the virus particles, the amount was very similar to that at the adjacent cell surface in the case of FPV (Fig. 5), but was sometimes greater around NDV particles (Figs. 6 and 7).

No staining of either cells or virus was found in the cytochemical control preparations.

![Fig. 3. Electron micrograph of thin section of NDV-infected HeLa cell. Fixation and staining as in Fig. 1-2. Spherical (right of field) and elongated (left) NDV particles. As with FPV (see Fig. 2), there is a limiting membrane covered by an amorphous zone, and a relatively empty central area. X180,000.](http://jb.asm.org/).
FIG. 4-7. Electron micrographs of thin sections cut through HeLa cells infected with FPV or NDV. Fixation and staining as in Fig. 1-2.

Fig. 4. Detail of a NDV bud forming at the surface of a cell; the cell membrane crosses the left of the field from above down, and, where it is thrown up to form the bud, it is altered by the addition of an outer amorphous layer. A complete particle, about 120 m,u in diameter, lies in the lower central part of the field below a cell process. X195,000.

Fig. 5. Detail of a FPV-infected cell after cytochemical staining. The cell membrane crosses the field from the left to the lower right side and shows very considerable adenosine triphosphatase activity; a spherical FPV particle (arrow) associated with the membrane has similar enzyme reaction product lying against its outer surface. X90,000.

Fig. 6 and 7. Small areas at the surface of NDV-infected cells stained cytochemically for adenosine triphosphatase activity. In both cases the cell membrane with associated enzyme reaction product crosses the center of the field; spherical NDV particles associated with these active cell membranes likewise carry the enzyme activity and have dense deposits of reaction product around their outer membranes. X90,000.

Discussion

The viruses used in the present work were selected to have a representative, which multiplies in HeLa cells, from each of the two subgroups into which myxoviruses have been divided (Watson, 1962). In these HeLa cell cultures, both agents behaved in a manner characteristic for their particular subgroup, the FPV producing an abundance of long viral filaments and relatively scanty rounded forms (Fig. 1 and 2), and the NDV producing proportionately far fewer elongated particles which were considerably shorter in length (Fig. 3). Furthermore, although the two agents multiply at markedly different rates, the cytopathic change which occurred during their periods of release was not accompanied by disruption of all normal cellular functions. This was shown by the finding that the HeLa cell cultures in which maximal amounts of FPV or NDV were being produced continued to possess the normal distribution of surface adenosine triphosphatase activity. An exactly similar preservation of this cell membrane enzyme function has already been noted during herpes infection of
these cells (Epstein and Holt, 1963a) and with fowl myeloblasts releasing an avian leukemia virus (Novikoff et al., 1962).

The finding of adenosine triphosphatase activity at the surface of both rounded and elongated particles of the two myxoviruses used in the present experiments fits well with current ideas on the formation of the outer viral envelope and mode of release by budding of membrane-bound viruses (Epstein, 1962b; Hummeler, 1963). It has long been believed on morphological grounds that myxoviruses are assembled at the cell surface whence they mature and escape by budding (Morgan et al., 1956, 1961; Schafer, 1963); the close correspondence between the presence or absence of adenosine triphosphatase reaction product around extracellular virus, and at the surface of the cells with which it was associated, provides clear functional confirmation that the cell membrane actually does form the viral envelope, and demonstrates the cellular site from which this viral enzyme is derived. In myxovirus infection, the cell membrane involved in the budding shows considerable morphological, antigenic, and functional alterations (Morgan et al., 1956, 1961; Marcus, 1962); yet, at the same time it must be sufficiently close to normal unaltered membrane for such a normal plasmalemmal function as adenosine triphosphatase activity to persist in it even when it has detached from the cell and become viral envelope. It would appear, therefore, that there is a virus-determined change in normal cell membrane used to form virus envelope, as was suggested by Hoyle (1962), rather than a synthesis of new virus-specific material unrelated to normal plasmalemma.

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Literature Cited


