Morphogenesis of Influenza A Virus in Ehrlich Ascites Tumor Cells as Revealed by Thin-Sectioning and Freeze-Etching

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The budding of a tumor-adapted strain of influenza A₀ virus at the surface of Ehrlich ascites tumor cells was studied by electron microscopy. Thin sections of budding sites showed the formation of a fuzzy coat on the outside of the cell membrane and simultaneously the apposition of a dark layer on the inner side. The continuity of cellular and viral membrane seemed to be preserved up to the point where the virion remained attached by only a thin stalk. Freeze-etching of virus budding sites yielded pictures in which a clear differentiation between the viral membrane and the host cell membrane was visible. The breaks across the fuzzy coat revealed striations corresponding to the "spikes" seen in negative contrast, whereas tangentially broken virus particles were best interpreted by assuming that splitting occurred midway between the two outer layers of the envelope.

Some strains of influenza virus can be adapted to grow in transplantable mouse tumors (1, 14) where they induce oncolysis (15). Viral oncolysis leads to the development of solid immunity to transplantation of the tumor (16). The immunogenic stimulus is thought to reside in certain host cell components which become incorporated into the virion as it emerges from the cell (18).

Since previous observations on the morphogenesis of influenza virus were largely restricted to the chick chorioallantois (7), it seemed of some importance to investigate the development of the virus in ascites tumor cells. In addition to conventional thin-sectioning, we applied the technique of freeze-etching, which seemed well suited to a virus maturing at the cell membrane. Particular attention was paid to those aspects of viral development which make it appear plausible that parts of the host cell membrane are integrated into the virus coat.

MATERIALS AND METHODS

Virus. The WSA strain of neurotropic, tumor-adapted A₀ influenza virus, originally obtained from W. W. Ackermann (1), was the same as used previously (15-18). Infected ascites tumor homogenates were lyophilized in small volumes, sealed under vacuum, and kept at -20°C. Reconstituted ampoules contained 10⁷ EID₅₀ (50% egg-infecting doses) per ml.

Tumor. The strain of hypotetraploid Ehrlich ascites tumor (EAT) was the same as used in previous

studies (15-18). The tumor was routinely passaged at weekly intervals in inbred male A2G mice by intraperitoneal inoculation of 10⁶ washed tumor cells. The LD₅₀ of this tumor both for inbred A2G and random-bred ICR mice is close to 10 cells per mouse (16). Several passage levels of this tumor were stored at liquid nitrogen temperature with 10% glycerol. We have observed occasional contamination of serially passaged tumor with extraneous viruses and bacteria, manifested by irregular growth and partial solidification of ascites or early death of the animal. In these cases, the passaged line was discarded and a fresh line was started from frozen stock. The present experiments were done on the 184th to 196th passage levels of EAT in A2G mice.

Mice. Inbred A2G mice, derived from A2GfCFW/Lac obtained in 1965 from the Laboratory Animal Centre, Carshalton, Surrey, England, were used for tumor passage. Random-bred ICR mice were used for virus experiments as described below.

Inoculation of EAT with virus. On day 0, ICR mice received an intraperitoneal inoculation of 10⁶ washed EAT cells from a 6- to 10-day tumor obtained from an A2G mouse. On day 7, 10⁴ EID₅₀ of WSA virus from lyophilized stock were inoculated into the ascites. The virus-infected tumor was harvested on day 9, when partial solidification, probably due to the formation of fibrin, had occurred (15).

Infectivity titrations. Serial 10-fold dilutions of virus were prepared in phosphate-buffered saline, pH 7.2, and placed in an ice bath. Embryonated chick eggs, 10 to 11 days old, were inoculated allantoically with 0.2 ml of virus dilutions; four to six

eggs per dilution were used. Inoculated eggs were incubated for 3 days at 35°C and chilled, and the allantoic fluids were tested for presence of hemagglutinin by standard procedures. One EID₅₀ was the estimated dose inducing hemagglutinin formation in 50% of inoculated eggs.

Processing of infected tumor for electron microscopy. At the time of harvest, the virus-infected tumor consisted of solidified areas with pockets of ascitic fluid. Only the cells in the fluid phase were collected, immediately cooled to 0°C, and washed three times in cold phosphate-buffered saline, pH 7.2. In several experiments, one part of the tumor cell suspension was used for freeze-etching and the other part for thin-sectioning.

Thin-sectioning. The tumor cells were prefixed for 30 min at 0°C in a mixture of 3% glutaraldehyde and 3% acrolein in 0.05 M cacodylate buffer (pH 7.2). The material was washed thoroughly for 90 min with 0.1 M cacodylate buffer (pH 7.2) containing 0.18 M sucrose and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). The cells were then stored overnight at 4°C in 2% uranyl acetate and finally embedded in Epon-Araldite (20). Sections were cut with an LKB Ultratome III by use of a diamond knife and were stained with lead citrate (25).

Freeze-etching. Within 30 min after harvesting, the washed and cooled cells were treated for 10 min at 0°C in phosphate-buffered saline containing 25% glycerol and they were then centrifuged. A drop of this pellet was mounted on a 3-mm specimen holder, frozen in Freon 22, and freeze-etched by standard methods in a Balzers unit (21–23). Replicas were cleaned with 70% sulfuric acid, Eau de Javelle (alkaline hypochlorite), and distilled water.

The thin sections and freeze-etching replicas were examined with a Siemens Elmiskop I or a Philips EM 200 electron microscope.

RESULTS

Observations on thin sections. Most particles seemed to bud at the cell surface or within membrane-bound vacuoles immediately beneath the cell surface, which probably corresponded to transversely cut invaginations (Fig. 1). Particles entirely detached from the cell membrane were rarely seen, because washing eliminated most of the free virus. We observed the following stages in virus development.

(i) There was bulging of the cell membrane, with apposition of some ill-defined, fuzzy material on the outside and of a heavily stained layer on the inside of the unit membrane. Within the bulge, fragments of darkly staining material were frequently seen (Fig. 2).

(ii) Further protrusion of the bulge appeared, the outer fuzzy coat sometimes showing radial striations (Fig. 3).

(iii) The protrusion became elongated, with constriction to form a stalk. The continuity between cell membrane and the outer two dark

layers of the virus membrane was preserved. The dark material within the particle was often arranged in longitudinal strands (Fig. 4).

(iv) Rod-shaped particles were seen, measuring up to 70 by 130 nm and connected with the cell membrane by a very thin stalk. The electron-dense material within the particle was arranged in parallel longitudinal strands about 10 nm thick (Fig. 5).

The virus membrane differed from the cell membrane in two respects. It carried a fuzzy coat on the outside and consisted, on close inspection, of three dark zones separated by two light spaces. The outer two dark zones were each 3 nm thick and were separated by a light band, also 3 nm thick, thus forming a typical unit membrane which in many pictures appeared continuous with the unit membrane of the cell (Fig. 9 and 10). The third innermost dark zone was 2 nm thick and was separated from the second dark zone by a narrow light band measuring approximately 2 nm. Although this three-layered appearance (five-layered if the light bands are also counted) could be detected in a majority of particles, it could not be resolved satisfactorily in every instance. Figure 12 shows a cross-sectional view with good resolution, in which there is even an indication of repeating subunits in the innermost layer.

The organization of the nucleoprotein could not be clearly seen, although characteristic differences between transversely and longitudinally cut virions were apparent. The former showed several dark spots about 10 nm in diameter (Fig. 12), whereas the latter had a longitudinal arrangement of strands of electron-dense material also 10 nm thick (Fig. 9 and 10).

The changes at the cellular level were neither conspicuous nor have we studied them very carefully. Formation of excess microvilli may be one of the effects of viral infection. An uneven distribution of ribosomes, which did not seem to occur close to the membrane of infected cells, possibly reflected accumulation of viral nucleoprotein at the cell periphery (Fig. 1).

Observations made with freeze-etching. Cell organelles and microvilli were easily recognized (Fig. 6). The virus particles appeared as oblong bodies placed predominantly with their long axis perpendicular to the cell membrane (Fig. 7 and 8). The structure most readily seen was a smooth core measuring about 100 by 50 nm. This core seemed to be laid bare by removal of a fringed layer consisting of 16-nm long surface projections, the profile of which was clearly discernible in some pictures (Fig. 13, 15, and 18). At high magnification, an array of 4-nm subunits could be seen at the basis of the fringed layer described

FIG. 1-5. All plates are of Ehrlich ascites tumor (EAT) cells infected with influenza A₀ virus, strain WSA. Bar indicates 100 nm, except as otherwise marked.

FIG. 1. EAT cell producing influenza virus particles (Vp). Nucleus (N), mitochondria (M), microvilli (Mv) comparable with structures seen in Fig. 6. Cytoplasm shows a ribosome-free peripheral zone and a ribosome-rich central region. Thin section.

FIG. 2-5. Different stages of the budding process of influenza virus on EAT cells. Apposition of a fuzzy coat on the outside and of a dark layer on the inside of a membrane which is continuous with the cell membrane. Thin sections.

FIG. 6-8. Same as Fig. 1-5.

FIG. 6. EAT cell producing influenza virus particles (Vp). Nucleus (N) with pores, mitochondria (M), microvilli (Mv) comparable with structures seen on Fig. 1. Freeze-etching.

FIG. 7. Detail from Fig. 6, showing a microvillus with budding virus particles. Several particles are "acorn-shaped." Freeze-etching.

FIG. 8. Virus particles budding on a microvillus. Note irregular distribution of 10-nm particles over membrane. Freeze-etching.

FIG. 9-14. *Same as Fig. 1-5.*

FIG. 9. *Budding virus particles, one of them showing the radial arrangement of surface projections in the fuzzy coat particularly well (arrow). Thin section.*

FIG. 10. *Virus particles budding at cell surface. Note continuity of cell membrane with viral envelope. Thin section.*

FIG. 11. *Influenza virus particle. Negative staining.*

FIG. 12. *Influenza virus particle. Thin section.*

FIG. 13. *Influenza virus particle. Freeze-etching.*

FIG. 14. *Influenza virus particle. Shadow-casting.*

FIG. 15-18. Same as Fig. 1-5.

FIG. 15. Surface view of a piece of cell membrane with budding virus particles. Insert: higher magnification of a particle (arrow). Freeze-etching.

FIG. 16. Budding particles on cell membrane. Note numerous 10-nm particles over cell membrane and their absence over the virus.

FIG. 17. The freeze-etching procedure has exposed the core of particle a and has enucleated particle b. Note fringed layer with radial striations and an array of 4 nm subunits at its base (arrows). Freeze-etching.

FIG. 18. Overall view of a field with apparently free virus particles. Filamentous (f) and normal forms either exposed or enucleated (compare with Fig. 17) are distinguishable. Freeze-etching.

above and immediately apposed to the smooth core (Fig. 17). Many particles showed a slight transverse constriction, giving them the appearance of acorns (Fig. 7 and 13). Only a few short filaments could be seen (Fig. 18); the virus strain used rarely produces filaments in any system.

We were not able to identify particles fractured in such a manner as to display a surface view of the fringed layer. The fracture either exposed the smooth surface of a body lying beneath the fringed layer or enucleated this body, thus revealing a concave surface of coarser structure (Fig. 17). However, parallel striations, which might have been expected if molding of the surface by the nucleoprotein core had occurred, were never seen.

The surface view of a piece of EAT cell membrane studded with budding virus allowed a comparison of the 10-nm particles, which are observed on nearly all cytomembranes (22), with similar particles on the virus membrane (Fig. 15 and insert; Fig. 16).

DISCUSSION

Negative staining has been widely used for the study of influenza virus (2, 9). This technique yields surface resolution unmatched by any other method presently available and sometimes reveals the arrangement of the nucleoprotein core of the virus (4, 13, 19). However, it is questionable whether the pleomorphism shown by negative contrast is due to distortions suffered during drying (24).

Preliminary studies on thin sections of Ehrlich ascites tumor cells infected with influenza A virus by Hurwitz had already revealed a remarkable uniformity in size and shape of budding particles (17). It may be that virus particles once liberated from the cell membrane change their shape somewhat, sinuous filaments becoming rather more rigid (11), rod-shaped particles becoming more or less spherical, and the "acorn-shaped" particles described above giving rise to the kidney-shaped particles often conspicuous in negative staining (Fig. 7, 11, and 13).

From a combination of data obtained by different preparation techniques (see for example, Fig. 11 to 14) and from the present results, the following picture of influenza virus can be attempted. The particle is surrounded by a fringed layer approximately 12 nm thick which, in thin sections, appears as a fuzzy coat containing radial spikes. Since this structure is not limited by a membrane on the outside, it is not altogether surprising that the fracture plane of freeze-etching breaks this layer transversely, following the radial striations, rather than tangentially. After break-

ing through the fringed layer, the fracture line is deflected tangentially by an obstacle, thereby revealing the smooth body lying beneath the fringed layer like a chestnut within its bur. The same analogy can be used to interpret pictures obtained by shadow-casting (Fig. 14), where the envelope appears to be shriveled around a core.

The exact location of the fracture plane at cell membranes is still controversial (6). According to our observations, the fringed layer surrounding the virus membrane is broken away. However, this fringed layer is at least 16 nm thick in freeze-etching preparations, rather than the usually accepted 12 nm obtained through other techniques. It appears that a rim of approximately 4 nm is added at the basis of the fringed layer proper. This rim is indicated by arrows on Fig. 17 and is probably part of the virus membrane. Hence, we are led to assume that the complex viral membrane fractures between its two outer dark layers, as indicated schematically in Fig. 19.

There is no doubt that the cell membrane is specifically altered before it is converted into a viral envelope, as evidenced by cytohemadsorption (10) and antigenic change (7). These alterations include the apposition of a fringed layer on the outside and of an additional layer on the inside. Morphological studies on other strains of influenza A virus (5) fail to differentiate clearly between those parts of the viral membrane which, in budding particles, are continuous with the host cell membrane and the third layer described above. Thin sections do not show any characteristic changes of the viral membrane. In freeze-etching, however, the exposed viral membrane surfaces are devoid of the particles characteristic of cell membranes (Fig. 15). This difference seems to occur early in the budding process and could be due to the fracturing of the viral membrane along its central plane, whereas the fracturing

FIG. 19. Schematic representation of viral envelopes. (1) Fuzzy layer with spikes; (2) three-layered "unit membrane" continuous with cell membrane; (3) inner apposition; (4) virus core; (5) fracture plane of freeze-etching; (6) 4-nm rim at basis of fringed layer.

plane of the surrounding cellular membrane would occur along its external, particle-covered surface (26). It is hoped that further studies will shed some light on the nature of these particles.

The arrangement of the nucleoprotein core could not be revealed by freeze-etching. Hoyle (12) has suggested that the space between the nucleoprotein helix and the virus envelope is usually tightly filled with hemagglutinin in the gel phase. This apparently results in a body too compact to be broken. Instead, it is either exposed by removal of the fringed layer or enucleated (Fig. 17). Since in negative contrast vast numbers of particles have to be scanned in order to find one which displays the nucleoprotein helix (2), it might still be possible by careful scrutiny of many pictures to detect an exposed nucleoprotein in freeze-etching.

Thin-sectioning involves fixation, dehydration, embedding, cutting, and staining, all of which are liable to introduce some distortion. Freeze-etching, on the other hand, is probably attended by the least amount of alteration and, although the best resolution is somewhat below that of other techniques, it may present the most "authentic" picture of virus in situ. Moreover, two entirely different techniques are unlikely to introduce identical artifacts. Since the freeze-etching results were obtained without prior chemical fixation, the overall agreement between conventional thin-sectioning and freeze-etching is particularly significant.

The continuity between cell membrane and viral envelope is clearly shown in many pictures (Fig. 2 to 5, 9, and 10). It is therefore easily conceivable that components of the cell membrane become incorporated into the mature virion. Evidence for incorporation is very strong for lipids (13) and mucopolysaccharides (8), and indirect evidence for incorporation of cellular surface antigens in the system used above has been offered previously (18).

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