Morphogenesis of Newcastle Disease Virus in Chorioallantoic Membrane

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Chick embryo chorioallantoic membrane, infected with the Blacksbury strain of Newcastle disease virus, was examined with an electron microscope to investigate the sequence of viral-induced host cell alterations. These were evident mostly in the endodermal epithelial cells lining the allantoic sac and were divided arbitrarily into three stages. Stage 1 was characterized by commencement of cell hypertrophy and hyperplasia and presence of fewer cytoplasmic inclusion bodies normally found in the cells; in stage 2, juxtanuclear nucleocapsid-glycogen aggregates appeared, and there were increased numbers of microvilli; stage 3 was characterized by increased cytoplasmic density and evidence of viral assembly and release. The morphological features of viral assembly and the virion are also described.

Most morphological descriptions of Newcastle disease virus (NDV) have been based on electron microscopy studies utilizing negative staining techniques (8, 13, 23, 24). There have been no detailed descriptions of the viral fine structure and assembly in sectioned material since the early work of Bang (1), who reported evidence of viral release from a modified cell surface and some alterations in endodermal epithelial cells of chick embryo chorioallantoic membrane (CAM) after infection with NDV. The purpose of this report is to extend the earlier observations and to describe some of the finer details of cellular alterations and viral morphogenesis made possible by more refined modern preparative techniques of electron microscopy and the improved resolution and magnifications available today.

MATERIALS AND METHODS

The Blacksbury strain of Newcastle disease virus was generously supplied by Vincent Groupé. Stock virus was prepared by injection of 10-day chick embryos in the allantoic sac with approximately 10^6 50% infectious doses (ID_{50}) of virus. Infected allantoic fluid was harvested after 48 hr at 37 C and stored at -65 C. Stock virus contained 10^8 ID_{50} /ml. For electron microscopy, samples of CAM were fixed overnight at 2 C in a phosphate-buffered modified Bouin fixative (26). The tissues were washed subsequently with the phosphate buffer, treated with a 1% solution of osmic acid in phosphate buffer for 1 hr, washed again with the buffer, dehydrated, and embedded in Maraglas (10). Thin sections were stained with uranyl acetate (25) followed by basic lead citrate (22) and examined with an electron microscope (Norelco EM 300) at an accelerating voltage of 80 kv.

RESULTS

The Blacksbury strain of NDV was chosen for these studies, because it is attenuated virus which permits prolonged survival of infected cells, in an attempt to determine an orderly sequence of events. At 10 days of development, chick embryos were infected with 10^6 ID_{50} of the Blacksbury strain of NDV by injection into the allantoic sac, and infected and uninfected control embryos were incubated at 37 C. Infected and control eggs were removed at various intervals after injection (16, 25, 48, 72, 90, 95, and 114 hr), and pieces of CAM were processed for electron microscopic examination.

The allantoic sac of 10-day chick embryos is lined with approximately 10^8 cells (12). Each egg was infected at this age with 10^6 ID_{50} of virus, therefore infection was asynchronous. Thus, at each time interval, cells were evident at various stages in the viral growth cycle. Under these conditions, the exact sequence of events could not be determined. However, trends in the overall sequence were obvious and the morphological events after infection were divided arbitrarily into three stages.

Cell alterations. The most obvious morphological alterations resulting from infection of the allantois were hyperplasia of the epithelial cells, as described earlier by Bang (1, 2), and changes in their cytoplasmic composition. The latter were particularly manifested in the layer of epithelial cells lining the allantoic sac. The fine structure of endodermal cells of the CAM has been described in detail by Borysko and Bang (6) and Leeson

and Leeson (16). The uninfected allantoic membrane consists of one or two layers of flattened epithelial cells containing the usual cytoplasmic organelles (Fig. 1). The luminal surface of these cells is relatively smooth with few microvilli. A prominent feature of these cells at 10 days of incubation is the presence of dense, membrane-bounded inclusions composed of finely packed granular material.

The earliest viral-induced alterations (stage 1) in the allantoic membrane were seen 48 hr after infection; among these were epithelial hypertrophy and hyperplasia. By 114 hr after infection, the epithelial lining increased from a control

FIG. 1. Endodermal cells of allantoic membrane from 15-day-old control chick embryo. Note scarcity of microvilli and presence of electron-dense granular inclusions (i) in cell lining the allantoic cavity (AC). $\times 11,200$.

maximum of two cell layers to four or five cell layers. The most obvious cytoplasmic alterations at this stage were noted in cells lining the allantoic cavity. In some cells there was a marked increase in the number of microvilli extending into the allantoic sac (Fig. 2), as reported previously (1, 3). The characteristic electron-dense granular inclusions were fewer and less dense, and contained loosely packed granular substance (Fig. 3).

The second phase (stage 2) was characterized by the consistent appearance in the vicinity of the nucleus of large accumulations of particles (Fig. 4) with the morphology and staining characteristics of glycogen (21). These particles measured 15 nm in diameter and stained faintly with uranium salts and heavily with lead. Similar particles were seen randomly scattered in the cytoplasm of comparable control cells, but not in the concentrated clusters characteristic of infected cells.

At about the same time, also in the vicinity of the nucleus, aggregates of filamentous structures were observed (Fig. 4). These measured 10 to 13 nm in diameter, and had transverse striations with a periodicity of 5 to 5.5 nm (Fig. 5). They are similar in appearance to the threadlike structures and filaments that have been described in tissue culture cells infected with viruses of the mumps-NDV-parainfluenza group and which are believed to be the viral nucleocapsids (5, 17, 18). In many cases, the filamentous aggregates were found in close association with large clusters of glycogen granules, and frequently mitochondria were seen immediately adjacent to this glycogen-filament complex (Fig. 4).

The most significant feature of stage 3, which was observed 72 hr after infection, was viral assembly and release (Fig. 6). Cells which were actively producing and releasing viral particles showed greatly increased cytoplasmic density due to the presence of large numbers of free ribosomes and diffuse accumulations of the filamentous material described above, which we believe to be the viral nucleocapsid. Whereas earlier this filamentous material was in juxtannuclear aggregates, it now appeared mostly concentrated subjacent to the luminal surface. The glycogen granules that previously were clustered and in close association with the juxtannuclear filamentous aggregates were now scattered within the cytoplasm. At this stage, the microvilli appeared still more numerous and more prominent than in the previous stage, and they were longer and frequently forked or branched.

Viral assembly. Viral subunits were assembled either as filaments or spheres. In each case the morphogenesis of NDV resembled that described

FIG. 2. Epithelial cells of allantois from 12-day-old chick embryo 48 hr after infection with the Blacksburg strain of NDV. In cell layer lining allantoic cavity (AC), note proliferation of microvilli and hypertrophy in cell at left, and unaltered appearance of cell at right. $\times 6,400$.

FIG. 3. Part of endodermal cell lining allantoic sac from 14-day-old chick embryo 95 hr after infection with NDV. Several granular cytoplasmic inclusion bodies of low electron density (arrowheads) are visible below the cell surface (compare Fig. 1). $\times 27,900$.

FIG. 4. Endodermal cells lining the allantois of 15-day-old chick embryo 114 hr after infection with NDV. Aggregates of filamentous structures (F) and accumulations of glycogen particles (G) are in cytoplasm near nucleus (N). A similar aggregate in adjacent cell is at upper left. Allantoic cavity is to right. $\times 15,400$. Filamentous nature of aggregates and some glycogen particles are shown in inset, which is higher magnification of area A. $\times 37,650$.

FIG. 5. High magnification of a filamentous aggregate in endodermal cell lining allantoic cavity from 14-day-old chick embryo 95 hr after infection with NDV. Note periodic crossbanding of filaments (arrows). $\times 119,700$.

with parainfluenza viruses (4, 7). Typical budding forms of the Blacksburg strain of NDV characteristic of the filamentous and spherical types are shown in Fig. 7, 8, and 9. The viral envelope was derived from, and therefore continuous with, the host cell plasmalemma. Nevertheless, the viral envelope was clearly distinguished from the host cell surface by its greater electron density and by the presence of an extramembranous coat which corresponded to the filamentous projections seen in negatively stained preparations (8, 13, 24). The extramembranous coat delineated the limits of the budding virion and served to distinguish between profiles of viral particles and cross sections of microvilli. Nucleocapsid material was seen closely applied to the inner surface of the differentiating viral envelope.

Structure of the virion. In sections of NDV-infected CAM, viral particles were seen as spheres, pleomorphic forms, and long filaments (Fig. 10). The slightly ovoid spheres measured 70 to 140 nm along the major axis and 65 to 115 nm along the minor axis, and the extramembranous coat was between 10 and 13 nm thick. Other investigators reported the average size of the rounded particles as 100 (1) and 90 to 140 nm (9). The filamentous forms were variable in length but they had a uniform diameter of 75 to

100 nm along their entire length. Frequently they showed the presence of a clublike ending.

Most morphological descriptions of NDV have been based on studies of negatively stained preparations (8, 13, 23, 24). These indicated that the helical nucleocapsid was arranged as a randomly folded structure within the viral envelope. In contrast, sections of spherical NDV particles most often showed the nucleocapsid closely applied to the inside of the envelope, with a relatively electron-lucent core free of the dense nucleoprotein helix. Depending on the plane of section through the nucleocapsid filaments, the virion may appear to contain an array of dots inside the envelope (transverse sections of nucleocapsid), or to have a thick "inner membrane" (sagittal or oblique sections of nucleocapsid). Figure 11 shows a single particle with portions of the nucleocapsid oriented both transverse and longitudinal to the plane of section. This suggests that in fixed and sectioned material there is a more ordered arrangement of the nucleocapsid than appears by negative staining. A similar organization of nucleocapsid material within the virion has been shown in the related parainfluenza virus SV5 (7). In spherical particles the internal nucleocapsid was 14 to 16 nm in diameter. In the filamentous forms, the internal nucleocapsid

FIG. 6. Two adjacent lining cells of allantois from 14-day-old chick embryo 90 hr after infection with Blackburg strain of NDV. Cell at upper left shows dense cytoplasm, characteristic of later stages of infection when viral assembly and release are taking place. Free viral particles and budding forms are visible (arrowheads). Note presence of branched or forked microvilli (arrows). Adjacent cell is at earlier stage of infection. Note lower cytoplasmic density and lack of evidence of viral release. A filamentous aggregate (F), characteristic of earlier stage of infection, is seen at lower left. $\times 18,200$.

FIG. 7. Filamentous bud at luminal surface of a lining epithelial cell from allantoic sac of 14-day-old chick embryo 89 hr after infection with NDV. Continuity of viral envelope and plasmalemma of host cell is visible. Viral extramembranous coat ends at junction between the two (j). Section through nucleocapsid (n) is seen subjacent to viral envelope. $\times 135,900$.

FIG. 8 and 9. Spherical NDV budding from infected endodermal lining cells of chick allantois (from tip of microvillus, Fig. 8; and from cell surface, Fig. 9). Continuity of viral envelope and plasma membrane of host cell are evident. Note termination of viral extramembranous coat at junction (j) between virus and host cell, and orderly arrangement of nucleocapsid (arrowhead) subjacent to the viral envelope. $\times 71,800$.

FIG. 10. Virions within the cavity of chick embryo allantois infected with NDV. Note polymorphic nature of the virus (arrows). $\times 46,100$.

FIG. 11. A mature spherical NDV particle showing dense viral envelope surrounded by a less dense ex-membranous coat, and sections through nucleocapsid subjacent to viral envelope cut transversely and longitudinally. $\times 151,000$.

capsid material appeared less dense than in the spherical, was smaller in diameter (12 nm), and was arranged in a loose spiral subjacent to the viral envelope (Fig. 12). Thus, in both the spherical and filamentous forms, the nucleocapsid was concentrated directly beneath the viral envelope, leaving a relatively electron-lucent core. A similar arrangement has been described for parainfluenza virus type 2 (14).

DISCUSSION

The luminal border of lining epithelial cells of uninfected allantoic membrane characteristically contains membrane-bounded granular inclusion bodies (6, 11, 16). We observed that these bodies increased in number in older embryos, and it has been reported (16) that they disappear at 21 days of embryonic development, just prior to hatching. These structures are said to contain lipid (19), but otherwise their chemical composition and functions are unknown. The significance of the marked reduction in number and density of these inclusions after NDV infection is unknown.

Many of the viral-induced alterations of the allantoic epithelium suggest increased cellular activity. These include hypertrophy, hyperplasia,

increased cell surface area resulting from formation of microvilli, and accumulation of large aggregates of glycogen particles. It is interesting that, in cells which we have referred to as stage 2, there appeared to be a specific association of glycogen with mitochondria and the aggregates of filamentous material that we presumed to be viral nucleocapsid. These regions might be viral factory sites in which the glycogen has been modified as an energy source for viral synthesis.

The accumulations of filamentous material probably correspond to the basophilic ribonucleic acid-positive granules, described in the cytoplasm of NDV-infected cell cultures (15), which were shown to contain viral nucleoprotein antigen by fluorescent-antibody stain (20). Howe et al. (14) reported that similar filamentous aggregates in the cytoplasm of human cells infected with parainfluenza virus (type 2) were stained specifically with ferritin-labeled antibodies to viral ribonucleoprotein. These filaments first appeared as aggregates in the vicinity of the nucleus and later, when active viral assembly was proceeding, they were concentrated just beneath the cell surface as though they were transported to that site for incorporation into the virion. There is, however, a discrepancy between the dimensions and density of these filaments in the cytoplasm and in the spherical virion. The cytoplasmic filaments were less dense and measured 10 to 13 nm in diameter; those within the viral envelope were denser and thicker (14 to 16 nm). The simplest explanation for this is that, during incorporation within the virion, the helical nucleocapsid becomes condensed and thickened, possibly by unwinding of the helical structure, in a way similar to the mechanism of chromosomal condensation. The nucleocapsid strands within the filamentous virions, on the other hand, resembled more closely the cytoplasmic strands in density and dimen-

FIG. 12. Section of filamentous form of NDV cut close to its surface, showing loose spiral arrangement of nucleocapsid. $\times 120,800$.

sion. Presumably in the filament there would be no need for condensation.

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