Morphogenesis of a Cytomegalovirus from an American Bison Affected with Malignant Catarrhal Fever

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SUMMARY

A herpesvirus isolated from several organs of an American bison affected with malignant catarrhal fever was cultured in bovine foetal spleen cells and studied by electron microscopy. The fine structural features of the mature virion and the mode of virus morphogenesis were found to be similar to herpesviruses classified in the subgroup cytomegalovirus. The capsids were granular, hexagonal in shape and contained pleomorphic cores in thin sections. Envelopment of the capsids occurred primarily by budding on cytoplasmic membranes which appeared to be formed as extended vesicles of the Golgi apparatus; budding on nuclear membranes was only rarely observed. Cytoplasmic inclusions consisting of granular threads and amorphous electron-dense material were found in association with virions during the late stages of infection. The formation of cytoplasmic inclusions, the morphogenesis and ultrastructure of the virus are all consistent with classification of this virus as a cytomegalovirus.

INTRODUCTION

Malignant catarrhal fever (MCF), a highly fatal disease of cattle, bison, buffalo and deer, is characterized by lymphoid angiitis and multisystemic epithelial lesions (Plowright et al., 1960; Liggitt & DeMartini, 1980a, b). In Africa, MCF is caused by bovid herpesvirus 3, a cell-associated virus with cell-fusing activity. Bovid herpesvirus 3 is transmitted to cattle by wildebeest (Plowright et al., 1965). The viral cause of the MCF affecting cattle, bison and deer in countries other than Africa remains elusive. Elaborate virological investigations of clinical specimens from MCF-affected cattle using cell-associated and cell-free virus isolation techniques yielded bovine syncytial viruses, morbilli-like viruses and herpesviruses (Storz et al., 1976; Coulter & Storz, 1979). These agents did not induce MCF in limited attempts to reproduce this disease experimentally in calves or deer. In contrast to bovid herpesvirus 3, the herpesviruses isolated from cattle with MCF in Europe and North America were initially cell-associated, but after a few passages could be passed by cell-free fluids (Liebermann et al., 1967; Storz, 1968). These strains lacked cell-fusing activities. In this report we describe the morphology and morphogenesis of a herpesvirus isolated from an MCF-affected American bison, Bison bison. The ultrastructure and morphogenic features of this herpesvirus are consistent with classification as a cytomegalovirus.

METHODS

Cell cultures and virus. Primary cultures of bovine foetal spleen cells (BFS) were prepared according to Malmquist et al. (1969). After three passages in Eagle's minimum essential medium (MEM) containing 10% lamb serum, 500 µg streptomycin and 500 units penicillin per ml, the MEM was replaced with lactalbumin vitamin medium containing 10% heat-inactivated foetal calf serum and the same antibiotics. Cells were used in the 4th to 10th subpassage.

The virus strain we describe, designated as 75-P-2756, was isolated from an American bison with clinical signs of MCF. The virus was recovered by direct cultivation of cells from the thyroid, adrenal glands and the spleen of the affected bison. Cocultivation of the trypsin-dispersed cells from these organs with BFS cells also yielded this type of virus.
**Results**

*Electron microscopy.* For analysis by electron microscopy, monolayers of BFS cells grown in Falcon Petri plates were infected with virus stock. Cultures of virus-infected and uninfected control cells were taken at 24, 36, 48 and 72 h after infection and processed *in situ* according to the method of Brunk & Ericson (1972) by fixation for 1 h at 0 °C in 0.1 M-sodium cacodylate–hydrochloride buffer pH 7-4, containing 0.1 M-sucrose and 2% glutaraldehyde. The fixed monolayers were rinsed in 0.1 M-cacodylate buffer pH 7-4, post-fixed for 45 min in the same buffer containing 1% osmium tetroxide, and rinsed in 0.1 M-cacodylate buffer pH 7-4. After gradual dehydration in ethyl alcohol, the cell monolayers were separated from the plastic Petri dish with propylene oxide, pelleted, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT2-B ultramicrotome, then stained sequentially with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope.

For negative staining, infected monolayers were scraped from the Petri dish and centrifuged at 800 g for 10 min. The cells were suspended in a few drops of distilled water and centrifuged at 3000 g for 20 min. Small drops of supernatant were placed on formvar and carbon-coated grids, stained with 2% phosphotungstic acid (PTA) and dried under vacuum prior to examination.

**Morphology of virions**

The ultrastructural morphology of mature virions was established in thin sections of host-cell vacuoles, where they accumulated in large numbers (Fig. 1a). A dense core, varied in morphology and staining intensity, was detected within the capsid of the virion. Occasionally, empty capsids were observed. The capsids formed a hexagonal frame around the core. The structural features of the capsids from the viral isolate 75-P-2756 were also studied by negative staining (Fig. 1b) and found to possess ordered arrangements of short tubular capsomeres. The electron-dense PTA stain filled both the centre of the tubular capsomeres and collected outside on the surface of the virion. When viewed face-on towards the centre of the virion, the
Fig. 2. Morphogenesis of nucleocapsids in the nucleus of infected cells. Completed capsids extend in uniform rows from granular fibrils (F). Many of the capsids appear empty and are presumably defective (24 h post-infection). Linear arrays of nucleocapsids (arrows), capsid (arrowhead) and core (C) are shown. Bar marker represents 250 nm.

Fig. 3. Formation of granular fibrils in the cytoplasm of infected cells (15 h post-infection). The granular fibrils appear both as linear threads (arrows) which extend from ribosomes, and as paracrystalline structures (P). Bar marker represents 500 nm.

capsomeres appeared as small rings. When the view was towards the periphery of the virion, the sides of the capsomeres were observed.

An integument zone of moderate electron density was observed between the capsid and the viral envelope. Peplomeric projections of the envelope were detected as the most external structure of the virion. Mature virions were detected within the membrane-bound cytoplasmic vacuoles and in the cell culture fluid, but they were not found free within the cytoplasm of infected cells.

Capsid morphogenesis

Ring-like or hexagonal capsids, formed within the nucleus, were frequently observed in linear ordered arrays (Fig. 2). Assembly appeared to proceed by shaping of thin granular fibrils to form the ring-like capsids. In thin sections the capsids were granular and hexagonal in shape. The formation of the capsid most commonly occurred in association with electron-dense material, which evolved as the core of the virion. The evolving cores had different forms and different densities of staining. Empty capsids were also detected. In addition to the granular fibrils located in the nucleus and associated with capsid formation, granular fibrils of similar morphology were also observed in the cytoplasm, frequently extending from ribosomes as linear threads or as closely knit paracrystalline aggregates (Fig. 3). The appearance of the pattern of fibrils in the aggregates varied, probably depending on the angle of sectioning. These structures were never observed in uninfected cells. During assembly of the capsids, the cell nucleus and nuclear membranes remained intact.

Envelopment of the virion

The envelope of this virus was rarely observed to be formed by budding of the nucleocapsid on the inner nuclear membrane (Fig. 4). Commonly the capsids were found free in the cytoplasm of infected cells and obtained the envelope by budding on membranes of extended vesicles of the Golgi apparatus or the smooth endoplasmic reticulum (Fig. 5). Large membrane-bound
Fig. 4

Fig. 4. Envelopment of the virion on the inner nuclear membrane (arrow) (48 h post-infection). Bar marker represents 250 nm.

Fig. 5

Fig. 5. Envelopment (E) of virions on cytoplasmic membranes. Nucleocapsids are found free in the cytoplasm of the infected host cell (arrow). Only enveloped viruses were found within the membrane-bound compartments (48 h post-infection). Bar marker represents 200 nm.

Fig. 6

Fig. 6. Late stages of viral morphogenesis. Numerous enveloped virions are within membrane-bound cytoplasmic compartments (48 h post-infection). Bar marker represents 500 nm.

Fig. 7

Fig. 7. Cytoplasmic inclusion formed in late infection. These inclusions contain granular fibrils (F), electron-dense amorphous material (A), nucleocapsids (NC), and enveloped virions (EV). Bar marker represents 500 nm.
accumulations of virions were formed through this envelopment process in the cell cytoplasm (Fig. 6).

**Characteristics of cytoplasmic inclusions**

Most interesting electron-dense inclusions developed in the cytoplasm at 48 to 72 h of infection (Fig. 7). This type of inclusion was only observed in cells containing many virions. Although the shape of these inclusions was variable, they were always found outside the infected nuclei and had similar ultrastructural features consisting of an amorphous electron-dense substance, granular fibrils, and viral capsids with and without cores. These viral structures were found at the boundaries of the amorphous and fibrillar material. Enveloped virions were frequently associated with these cytoplasmic inclusions.

**DISCUSSION**

The virus strain 75-P-2756, isolated from a bison with clinical signs of MCF, replicates in the nucleus of infected cells, produces cytoplasmic inclusions, and possesses a morphology consistent only with the herpesvirus family (Becker *et al.*, 1965). Tubular capsomeres characteristic of herpesviruses were always observed on electron microscope examination of negatively stained preparations. The size of the capsids of this isolate, as measured in electron micrographs of thin sections, is approximately 100 nm. Examination of the capsid revealed a distinctly granular or beaded substructure which was observed with cytomegalovirus capsids, as contrasted with the smoother substructure of capsids reported for herpes simplex virus (Becker *et al.*, 1965; Iwasaki *et al.*, 1973; Smith & DeHarven, 1973). The remarkable variations of virion morphology reported for varicella-zoster virus were not observed with this isolate (Cook & Stevens, 1970).

One of the most interesting features common to cytomegalovirus was the formation of electron-dense cytoplasmic inclusions. The morphology of the inclusions was variable (Craighead *et al.*, 1972; Sarov & Alsady, 1975). Human cytomegalovirus isolates usually form amorphous membrane-bound inclusions approximately 300 nm in diameter. These inclusions have been isolated and shown to contain most of the antigens present in the cytomegalovirus virion, but they lack the presence of the DNA genome. Electron-dense cytoplasmic inclusions formed by cytomegalovirus isolates from animals are not completely surrounded by membrane. Capsids with cores are frequently observed at the periphery or scattered inside the electron-dense matrix of the cytoplasmic inclusion induced by isolates from animals (Fong *et al.*, 1980). The cytoplasmic inclusions formed by the bison isolate 75-P-2756 also contained capsids with cores associated with the electron-dense amorphous matrix and, in addition, they contained granular fibrillar material consistent in ultrastructure with the fibrillar material associated with capsid formation. Whether or not the cytoplasmic inclusions observed represent a second mechanism of assembly or are the result of breakdown of previously formed virions cannot be determined from these experiments.

Many features of the viral isolate 75-P-2756 from bison, such as capsid morphology, process of maturation by budding through nuclear or smooth cytoplasmic membranes, and the presence of cytomegalovirus-like cytoplasmic inclusions characterize this virus as a bovid cytomegalovirus and separate it from the bovid herpesvirus 3 of the antelope-associated form of MCF (Plowright *et al.*, 1965; Castro & Daley, 1982). The wide range of animal species naturally infected with cytomegalovirus (Weller, 1971) is thus further widened to include the bison.

Despite many attempts, a virus similar to bovid herpesvirus 3 has not been isolated from cases of MCF in Europe or North America (Storz *et al.*, 1976). Either viruses different from bovid herpesvirus 3 are involved as causative agents of MCF outside Africa where antelope species are intermediate hosts or detection of the putative bovid herpesvirus 3 is obscured by multiple infections with other types of herpesvirus such as cytomegaloviruses. The cultural and plaque characteristics of the herpesviruses cultured from MCF-affected cattle in Germany and the United States reflect cytomegalovirus properties (Liebermann *et al.*, 1967; Storz, 1968).
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