Biogenesis of Marek’s Disease (Type II Leukosis) Virus In Vitro: Electron Microscopy and Immunological Study

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The kinetic events involved in Marek’s disease herpesvirus infection of avian cell culture were investigated by assaying viral infectivity and antigenicity as well as by electron microscopy during the infectious cycle. The levels of viral infectivity and complement-fixing (CF) antigens revealed that the rates of appearance of infectious particles and CF antigens were not synchronous. Viral specific CF antigen could be detected 5 h after infection, whereas viral infectivity or the appearance of viral particles could be demonstrated only after 10 h of infection. High proportions of the recovered CF antigens during the various stages of the infectious cycle were found to be soluble and did not sediment with the virus particles. Cytological analysis of the developmental stages of the JM virus-infected cells by thin sectioning and electron microscopy revealed that at 8 h small particles approximately 35 nm in diameter appeared in the cell nuclei. The appearance of nucleocapsids occurred at 10 h, and these were of varying shapes; however, all were approximately 100 nm in diameter. At approximately 18 h postinfection, mature virus particles were observed. Viral maturation of the immature particles occurred by the acquisition of envelope from the inner leaflet of the nuclear membrane or from the cytoplasmic membrane of the cell.

MATERIALS AND METHODS

Cell culture. The growth medium used for duck embryo fibroblast cell line (DEF) and primary chicken kidney (CK) cell cultures was medium 199 (14) supplemented with a 2 to 5% (vol/vol) bovine fetal serum, 10% tryptose phosphate broth, penicillin, streptomycin, and mycostatin at concentrations of 100 µg. 0.2 mg, and 25 µg/ml, respectively. The pH was adjusted to 7.2 with 0.75% sodium bicarbonate.

Virus. The JM strain of MD (25) was used after 30 to 34 passages in DEF cells. The infected monolayers were harvested directly in their media, pooled, and centrifuged at 10,000 × g for 15 min to sediment the cells. The supernatant was saved, and the pellet was resuspended in cold distilled water and disrupted by sonic treatment for 90 s at 30-s intervals in a Biosonik II A (Bronwill Scientific Inc., Rochester, N.Y.) sonic oscillator. The coarse cellular debris which resulted was sedimented by centrifugation at 10,000 × g for 15 min in the cold. The supernatants were combined and centrifuged at 100,000 × g for 2 h at 5 C (all centrifugations were carried in a Spinco ultracentrifuge with an angle head). To cushion the virus particles during their sedimentation, 2 ml of 2% (wt/vol) solidified Noble agar was placed in the bottom of the centrifuge tube. The pellet was resuspended in growth medium and filtered through a membrane filter (0.45 µm pore size; Millipore Corp.).

Marek’s disease herpesvirus (MDHV) is a lymphoproliferative disease of chickens that was reported to be caused by a herpes-type virus (4, 16). The growth of MDHV in cell culture results in the formation of cytopathic effects that are amenable to quantitative assay by microplaque count in cell culture (33). Hamdy and Sevoian (8) reported the development of specific complemen-fixing (CF) antigens in avian cell cultures infected with the JM strain of MDHV. Although detailed studies of virus development and morphogenesis in infected cells have been reported in herpes-type viruses (e.g., 1, 7, 19, 24, 26–28), to our knowledge the kinetic events involved in MDHV infection have not been reported. There is, however, a substantial literature dealing with the biological, morphological, and immunological characteristics of MDHV (e.g., 2, 3, 4, 6, 15, 17, 33). This paper is an attempt to describe the levels of infectivity and antigenicity during the infectious cycle as well as the morphogenesis of the virus in infected cells.

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This 0.45-μm filtrate represented the cell-free virus suspension.

**Microplaque assay in CK cell culture.** Monolayers of cultured CK cells were prepared in 60-mm plastic petri dishes and were used when confluent growth occurred, usually at 4 days. After removal of the growth medium, 0.2 ml of a serial fourfold dilution of the suspension to be assayed was inoculated onto the monolayers. Adsorption was permitted to occur over a 3-h period, during which time the dishes were rotated periodically. After 3 h, the medium was replaced and the cell cultures were incubated an additional 6 days at 37°C in an atmosphere of 5% CO₂-95% air. During this time, plaques appeared and were counted. Duplicate plates were used for each dilution and the titers were calculated in microplaque-forming units (PFU) by averaging the counts from each dilution giving between 30 and 120 plaques per petri dish.

**Kinetics of JM virus adsorption and development in DEF.** DEF were inoculated with 0.2 ml of JM virus suspension at a multiplicity of infection (MOI) of 4 PFU/cell, and were incubated at 37°C in a 5% CO₂-95% air atmosphere. Duplicate plates were removed at 1-h intervals for 6 h, and the cell cultures were washed with 9.8 ml of phosphate-buffered saline. Fourfold serial dilutions of the phosphate-buffered saline (containing the unadsorbed virus) were assayed for infectivity in CK monolayers as described above.

The levels of viral infectivity and antigenicity and the morphological events occurring during the infectious cycle were determined by the following experimental design. A pool of washed packed cells was suspended in JM stock virus yielding a mixture containing 2 x 10⁸ cells/ml with an input multiplicity of virus of 10 PFU. Adsorption was permitted to occur at 37°C, and the cell cultures were incubated at 37°C in 5% CO₂-95% air. At 4-h intervals, samples were removed for assaying viral infectivity by microplaque assay in CK cell cultures and titrating CF activity. At approximately 2-h intervals, samples were removed and prepared for thin sectioning and electron microscopy to study the morphogenesis of JM virus in infected cells.

**CF test.** JM virus-specific CF antigens were measured by the method of Hamdy and Sevoian (8) with the following modifications. The concentration of sensitized sheep red blood cells in Veronal buffer diluent (8) was decreased to 0.04% (vol/vol). The antigen titer was calculated from the dilution of antigen that fixed 50% of the complement, rather than 70%. These modifications increased the sensitivity of the test at least 1,000-fold.

**Electron microscopy.** Control and infected cell culture monolayers were fixed in glutaraldehyde and osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 (13) as previously described (F. Hamdy et al., Infect. Immunity, submitted for publication).

Thin sections were cut with a diamond knife on a Porter Blum MT-2 ultramicrotome (Sorvall, Inc.) and stained with uranyl acetate (10) and lead citrate (20). Electron micrographs were taken with a Phillips EM 200 electron microscope.

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**RESULTS**

**Kinetics of adsorption of JM virus.** The assay of the number of unadsorbed virus particles with time permitted the enumeration of the rate of attachment of JM strain to DEF. Approximately 60% of the JM virus adsorbed to DEF in less than 1 h (Fig. 1). Viral adsorption continued, but at a much slower rate, approaching 100% adsorption at approximately 4 h.

**Growth curve of JM virus on DEF.** To determine the time course of virus development, DEF cell cultures were infected with the JM strain at an MOI of 10 and assayed for infectivity and antigen induction with time (Fig. 2). Virus concentration as measured by PFU remained very low and at an essentially undetectable level during the first 10 h of incubation. At approximately 10 h, there was a rapid rise in titer from 0.04 PFU/cell to 17.0 PFU/cell at 24 h, representing a 425-fold increase in virus concentration. Concomitant with this rapid rise in the number of infectious virus particles was the rise in CF viral specific antigen. This antigen was undetected in the 4-h sample but thereafter increased rapidly from 0.36 units at 5 h to greater than 16 units at 18 h.

Interestingly, viral CF antigen and infectivity curves were not parallel. The difference in slopes is expressed by differences in the ratio of

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**FIG. 1. Adsorption rate of JM virus to DEF.**
infectivity (PFU/ml) to antigen (CF units/ml). This ratio was 0.1 in 10-h samples, whereas in 18-h samples it was 5.6. This suggests that during the course of virus infection, some of the antigen is not associated with virion structure. Evidence for the presence of noninfective antigen that is not part of the virus particles was revealed by centrifuging cellular homogenates that were collected during the course of the growth experiment. Samples of infected cells were harvested after 5, 10, 12, 18, and 24 h postinoculation and were sonically treated and centrifuged at 80,000 × g for 30 min. The supernatant fluids were carefully removed with a Pasteur pipette and centrifuged a second time at 80,000 × g for an additional 30 min. The resulting pellets were pooled and resuspended in 2-ml volumes of medium. The antigen and infectivity titers of the original cellular homogenates, pellets, and supernatant fluids from specimens at each time interval were determined (Table 1). As is evident, essentially all of the viral infectivity (99% or more) was found in the pellets, whereas only 6 to 18% of the antigen had sedimented with them. Thus, throughout the period of study, most of the antigen was situated in particles of relatively small size.

**Virus morphogenesis in infected cells.** When JM virus-infected cells were examined chronologically by electron microscopy of thin

sections, it was possible to observe herpesvirus particles in different developmental stages. Some of these stages are shown in Fig. 3 to 9. The earliest observable cytological changes in infected cells occurred at 6 h with the formation of both binucleate cells and giant cells with margination of the nuclear chromatin (Fig. 3). The latter changes occurred at 6 h postinfection before any virus particles could be detected by electron microscopy. It must be noted that viral specific CF antigen was demonstrable at this stage, and it is possible that the changes in the antigenic determinant of the membranes of the infected cells contribute to a positive CF test and to cell fusion. It was only at approximately 8 h that small particles, 35 nm in diameter, were observed in the cell nuclei (Fig. 4). These particles displayed six-sided symmetry and resembled the central cores of some of the capsids that were seen in later stages. The presence of the 35-nm particles is consistent with the hypothesis that their synthesis occurs within the nucleus. Capsids were approximately 100 nm in diameter, and their shape varied from round to hexagonal. Some of the capsids appeared empty, and some appeared double shelled, unsealed, or with a dense core (Fig. 5). However, in all cases, the capsids retained their shape and their overall diameter. Maturation of the virions occurred by acquisition of an envelope from the inner leaflet of the nuclear membrane (Fig. 6). In some thin sections, the nuclear membranes were observed to be differentiated into multiple units surrounding the capsids in the nucleoplasm. Thus the mature MD virion was approximately 180 nm in diameter and was composed of a complex envelope surrounding a capsid 100

![Fig. 2. Growth curve of JM virus in DEF. a, CF activity (arbitrary units); b, virus infectivity (PFU).](image)

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* The percentage of infectivity recovered in the pellet. The remainder is in the supernatant.
* The overall infectivity recovered in percent of the original material.
* The percentage of CF activity recovered in percent of the original material.
* The overall CF activity recovered in percent of the original material.
Fig. 3. Electron micrograph of thin section of DEF cell culture 6 h after infection with JM virus showing a binucleate cell. Note the margination of the nuclear chromatin material (arrows). N, nucleus; NI, nucleolus. Bar, 1 μm. All materials for thin-sectioning and electron microscopy were fixed with glutaraldehyde-osmium and stained with uranyl acetate and lead citrate.

Fig. 4. Electron micrograph of thin section of chicken kidney cell culture. Note the presence of small particles (P) approximately 35 nm in diameter. These particles first appeared at 8 h postinoculation and prior to the appearance of nucleocapsids. NM, nuclear membrane; N, nucleoplasm. Bar, 100 nm.
FIG. 5. Electron micrograph of thin section of CK cell nucleus 12 h postinfection with JM virus. The presence of small particles (P), cores without limiting membranes (C), and nucleocapsids with a single limiting membrane (Nc) are apparent. N, nucleoplasm. Bar, 100 nm.

FIG. 6. Electron micrograph of thin section of JM virus-infected DEF cell culture. The nuclear membrane (NM) differentiated and traversed the field, extending into the nucleoplasm as a membrane bound invagination containing several mature virus particles (MP). Each particle was enveloped by its characteristic outer membrane (diameter 180 nm). Two immature viral (IV) nucleocapsids are also visible together with the small particles (P); N, nucleoplasm. Bar, 100 nm.
Fig. 7. Electron micrograph of thin section of DEF. Nucleocapsids (Nc) are seen in the nucleoplasm. Several nucleocapsids are opposite to the nuclear membrane (NM). An unclosed nucleocapsid (arrow) is visible. A mature virus particle (MV) is shown in the cytoplasm (C) surrounded by a vacuole. Spikes are seen attached to the outer envelope of the virion; N, nucleus. Bar, 100 nm.

Fig. 8. Electron micrograph of a thin section of DEF infected with JM virus 24 h postinoculation. Mature virus particles (MV) are present extracellularly. Spikes attached to the outer envelope are apparent. C, cytoplasm; PM, plasma membrane. Bar, 100 nm.
FIG. 9. Electron micrograph of a thin section of kidney cell 18 h postinoculation with JM virus. Note that the nuclear membrane distorts its position changing the outline of the nucleus. Nucleocapsids (arrows) are seen in the nucleoplasm (N); NM, nuclear membrane; M, mitochondrion; ER, endoplasmic reticulum. Bar, 1 μm.
nm in diameter. Internal to this was the central core approximately 65 nm in diameter. The nucleic acid is believed to constitute the electron-dense inner zone. The viral envelope consisted of an external membrane that measured 20 nm thick. Beneath this external membrane there appeared an electron-translucent zone (Fig. 6) similar to what is proposed to be an inner envelope or inner membrane in herpesviruses (22, 23). The cores in the capsids and virions differed with respect to their staining affinity, appearing with different degrees of electron opacity (Fig. 5 and 7).

The virus particles were very rarely found in the cytoplasmic space, but when present in this region were usually in vacuoles (Fig. 7). It must be noted that virions that acquired their envelopes from the nuclear membrane (Fig. 6) were larger in size than the virions seen in the cytoplasm (see, e.g., Fig. 7; 180 versus 130 nm, respectively). Virions were rarely seen in the extracellular region, but when observed they appeared to contain spikes projecting from the surface of the outer envelope (Fig. 7 and 8). Several infected cells showed displacement of their nuclear membranes, changing the outline of the nuclei (Fig. 9).

DISCUSSION

Quantitative data during the infectious cycle of JM strain of MD virus showed that the rate of attachment of the virus to DEF was relatively slow and reminiscent of other herpesviruses (11). The production of viral-specific CF antigens several hours (approximately 5 h) prior to the recognition of the first infective particles points to the possibility that the viral components are being “programmed” or assembled early in the infectious cycle. Similar response of early antigen production was reported in herpes simplex virus infection (32). Moreover, the synthesis of this CF antigen was not at the same rate as the viral growth rate. Wildy and Watson (32) were able to show a definite difference in the rate of synthesis between the infectious particles and CF antigen, and recent work by Tarrow and Salvin (29) has established the presence of virus-specific, nonvirion antigen in herpes simplex virus 1- and 2-infected cells. It is possible that most of the CF antigen is situated either in structural proteins of the virion that are not incorporated into the particles with virus specific determinants or in enzymes, or other proteins, specified by the virus genome. Long and Velicer (P. A. Long and L. F. Velicer, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 204, 1973) have recently described a soluble antigen from MD-infected cells having a sedimentation coefficient of 3.5 to 4.0S, and they showed by polyacrylamide gel electrophoresis that this antigen was composed of at least five proteins. The formation of polykaryocytes in infected cells that also occurred before the recovery of virus infectivity or of virus particles may be related to the changes in the antigenic determinants of the membranes of infected cells and may possibly be initiated by these changes.

Roane and Roizman (21) demonstrated that the surfaces of cells infected with herpes simplex virus changed their antigenic specificity. Nii et al. (19) reported that the synthesis of infective virus is not necessary for the formation of polykaryocytes provided that the surface of the cell becomes altered. Furthermore, Heine et al. (9) have shown that the surface of infected cells contained, in addition to the normal cellular proteins, viral proteins integrated into the plasma membrane.

The observation of small 35-nm particles free in the nucleoplasm that resembled the central cores of some of the nucleocapsids indicated that replication of viral DNA preceded the formation of capsids. We routinely observed these small particles either in association with nucleocapsids or in their absence. However, the latter condition was observed only at early stages of infection, that is, prior to 10 h. Similar particles have also been observed in the cell nuclei of herpes simplex-infected cells (26, 27, 31), varicella-zoster virus (30), pseudorabies herpesvirus (7), and frog renal adenocarcinoma herpesvirus-infected cells (28). These structures were described as being either small particles, 35 nm in diameter (16), or dense or nuclear aggregates (28). Stackpole (28), in an electron microscope autoradiographic study of the development of the frog renal adenocarcinoma herpesvirus, used [H]thymidine-labeled virus to infect monolayers. He determined initial incorporation of [H]thymidine into dense granular aggregates only in infected cells at 4 h postinfection. This indicates that these structures contained newly synthesized viral DNA, which is consistent with the hypothesis that these particles were precursors of the central cores of the nucleocapsids and represent the viral genome. Roizman (22) proposed that these small particles were either aberrant forms of the core and inner capsid or a precursor of the core (the latter hypothesis explaining their accumulation). We tend to think that these particles were precursors of the deoxyribonucleic acid-protein cores, as they were the first virus forms to appear in
the infected cells, and also because of their association with the viral deoxyribonucleic acid (28).

The difference in shape of the nucleocapsids (polygonal, hexagonal, circular with rod-shaped core, or unclosed bizarre form) may be a result of variation in the plane of sectioning. In reference to the variation in electron density of the cores of the nucleocapsids, it is not clear whether these changes are essentially artifacts reflecting insignificant differences in permeability to fixative or staining solutions, or indicate significant, but as yet unknown, characteristics in the virus development. The variation in shape of the nucleocapsids has been observed to occur in all the known members of the herpesvirus family. For example, Schwartz and Roizman (24) demonstrated six different shapes of viral particles in HEP-2 cells infected with four strains of herpes simplex virus. Stackpole (28) also demonstrated variations in the shape of nucleocapsids seen in herpesvirus-infected frog tumor transplant cells. King et al. (12), in an ultrastructure study of nine agents of herpesvirus of monkeys, demonstrated at least four distinct morphological types of intranuclear, nonenveloped capsids with each of the nine agents.

The envelopment process either originated from the inner leaflet of the nuclear membrane or from the cytoplasmic membrane. This has been shared by many of the other herpesviruses (5, 24). Darlington and Moss (5) have very clearly shown that the maturation of the herpesvirus occurred essentially at the nuclear membrane and to a much lesser degree in the cytoplasm. Our study tends to confirm their observations. In reference to the spikes we observed (Fig. 7 and 8), it is possible that the cytoplasm may be a second site for the envelopment of the nucleocapsids. Similar results have been reported with other herpesviruses (1, 6, 7, 19, 28).

It is interesting to note the differences in size and structure of the virions whose maturation occurred at the nuclear membrane or in the cytoplasm. Similarly, Spring and Roizman (27a) observed that infectious virus extracted from the nuclei of infected cells differed from the viruses extracted from the cytoplasm with respect to size and degree of stability when centrifuged in solutions of high ionic strength. They also showed that the nuclear and cytoplasmic viruses differ in their antigenic determinants as shown by differing results in neutralization tests. In general, the basic morphology and morphogenesis of MDV substantially agreed with those described for other members of the herpesvirus group such as herpes simplex virus (19, 23), equine abortion virus (1), frog renal adenocarcinoma (28), and herpesvirus of monkeys (12).

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LITERATURE CITED