Electron Microscopy of a Herpes Virus Associated with the Agent of Marek’s Disease in Cell Culture

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SUMMARY

Duck embryo fibroblast cultures inoculated with blood from chickens infected with JM strain of Marek’s disease developed a cytopathic effect 11—25 days postinoculation. DNA containing intranuclear inclusion bodies were seen in many polykaryocytes associated with the cytopathic effect.

Some intranuclear granular bodies were seen in thin sections of these cells examined with the electron microscope. The granular bodies probably corresponded to the intranuclear inclusion bodies seen with the light microscope. Naked herpes virus-like particles and occasional particles with an additional membrane were found in the nuclei of infected cells. The naked particles appeared to start budding into the cytoplasm and nuclear vesicles and obtain an additional membrane in this process. The naked and enveloped particles were occasionally seen in the cytoplasm accompanied with some membrane-like structures. However, only typical naked particles were seen in negatively stained preparations of concentrated culture fluids. These particles measured 95—100 nm and had 162 capsomeres. The morphogenesis and morphology of the virus were similar to those of herpes-type viruses.

INTRODUCTION

Marek’s disease is an infectious lymphoproliferative disease of the domestic fowl characterized by lymphoid cell infiltration of the nervous system and by tumors in the viscera. The disease is transmitted readily by direct contact with infected birds and through the air (18).

In blood and tumor homogenates from infected birds, the agent has been found to be extremely sensitive to freezing and thawing, homogenization, and ultrasonic oscillation (3, 4). The agent can be removed from suspension by two cycles of centrifugation at 2,000 X g (4). Owen et al. (15) used sex chromosomes as markers and found that experimental transmission of the disease with whole-cell inocula was not due to cell transplantation. These observations may indicate the presence of an infectious Marek’s disease agent in cells, and that viable cells are required for survival of the agent and its infectivity. Extensive electron microscopic studies of blood and tumors from infected birds (K. Nazerian, unpublished data), however, has failed to demonstrate and identify the agent.

Propagation of the agent of Marek’s disease and production of a cytopathic effect in duck embryo fibroblast monolayer cultures was reported recently (21). A good correlation was obtained (14) between the occurrence of this cytopathic effect, the presence of herpes virus-like particles in infected cells, and the ability of these cells to reproduce Marek’s disease in susceptible birds. Similar results have also been obtained with kidney cell cultures derived from Marek’s disease-infected birds (5, R. L. Witter, unpublished data). The circumstantial evidence relating this virus to Marek’s disease and similarities of the virus to the disease agent strongly suggested the implication of the virus in the etiology of Marek’s disease (14).

This report further describes ultrastructural studies of duck embryo fibroblast cultures infected with Marek’s disease agent and describes some of the characteristics of the herpes-type virus seen in these cultures.

MATERIALS AND METHODS

Source of the Virus. Infectious blood from birds inoculated with JM strain (17) of Marek’s disease was used as the source of virus. Donor birds showed clinical signs of the disease and had gross lesions at necropsy.

Propagation of the Virus. The virus was propagated in monolayers of duck embryo fibroblast secondary cultures. The growth medium consisted of a mixture of four parts of Medium 199, and five parts of Medium F10 (Grand Island Biological Company) and contained 5% tryptose phosphate broth, 4% calf serum, and appropriate quantities of antibiotics. Cultures were inoculated with infectious blood as described earlier (21) and were incubated in humid atmosphere of 5% CO₂ at 37°C. Subcultures were made every three to six days. Some infected cultures were kept up to 20 days without any subcultures being made during this period.

Suspension Cultures. Monolayers of infected duck embryo fibroblast cultures showing extensive cytopathic effect were trypsinized, and the cells were suspended in 100 ml of growth medium to give a final concentration of 3 X 10⁵/ml. The cell suspensions were incubated in a 250-ml spinner flask (Bellco) on magnetic stirrer in a 5% CO₂ atmosphere at 37°C with rotation of the Teflon-covered magnet at about 100 revolutions per minute. Growth was judged by the rate of pH change and increase in cell population. Culture fluid was changed every 48 hours.

Acridine Orange Staining. Coverslip preparations from infected cultures showing extensive cytopathic effect were fixed

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in absolute alcohol and stained with 0.5% acridine orange in 0.01 M acetate HCl buffer pH 2.7 (1) for 30 minutes. The preparations were then rinsed in buffer, mounted in phosphate-buffered saline, pH 7.4, and examined with a light microscope using ultraviolet illumination.

**Concentration of the Virus.** Growth fluids were collected from infected cultures 48 hours after the appearance of cytopathic effect and centrifuged for 60 minutes at 112,000 X g. The pellets were then suspended in 1/150 of the original volume of distilled water, frozen and thawed twice, and clarified by low speed centrifugation. Negatively stained preparations of the resulting virus suspensions were made for electron microscopy with 2% potassium phosphotungstate pH 7.0 and 0.01% sucrose in distilled water.

**Electron Microscopy.** Monolayers of infected and control duck embryo fibroblast cultures were scraped from the Petri dishes and fixed in 1% osmium tetroxide. Large clumps of cells which formed in suspension cultures were sedimented and similarly fixed. All specimens were dehydrated in ethyl alcohol, embedded in Epon 812 (11), sectioned with an MT-2 Porter-Blum ultramicrotome, and stained with uranyl acetate (9) and lead citrate (10). All preparations were examined with a Siemens 1A electron microscope.

**RESULTS**

**Propagation of the Agent in Cell Culture.** In several experiments, 15 different monolayers of duck embryo fibroblast cultures prepared from different pools of embryos developed a cytopathic effect (21) 11–25 days after exposure to infectious blood from 15 different donor birds. Cytopathic effect did not develop in two cultures treated with blood from control birds or in any of the 15 uninoculated control cultures. The cytopathic effect could be passed repeatedly to new cultures by inoculation with whole-cell suspension or unfrozen culture fluid from infected cultures. The cell destruction associated with cytopathic effect was not extensive, and the number of morphologically altered cells in a focus increased only slightly in periods as long as 20 days. Foci of altered cells could be easily seen and counted in unstained preparations with the aid of an inverted light microscope. Foci of altered cells grown on coverslips and stained with acridine orange showed numerous polykaryocytes with as many as eight nuclei. The large inclusion bodies found frequently in the nuclei of such cells stained bright green, while the nucleoli and cytoplasm stained reddish orange (Fig. 1).

Three different infected monolayer cultures with extensive cytopathic effect were propagated in suspension. All grew well, as indicated by rapid change of pH and increase in turbidity. The large and small clumps of cells which formed as the cultures grew older were not easily dispersed, and it was thus difficult to obtain an accurate measure of cell growth. Small aggregates of cells and individual cells transferred to Petri dishes grew more slowly than cells in comparable cultures maintained as a monolayer, but the cultures eventually formed confluent monolayers with typical cytopathic effect.

**Electron Microscopy.** Herpes virus-like particles were found in all cultures inoculated with infectious blood, but not in those treated with control blood or in any of the control cultures. The incidence of virus-containing cells varied from about 1 to 10% depending on the line of cells and the age of the cultures. The number of altered cells and cells containing virus increased with continuous passage of monolayer cultures, and the proportion of cells containing virus was greater in suspension than in monolayer cultures. In one suspension culture, 83% of the aggregated cells and 30% of the nonaggregated cells contained virus particles compared with only 10% of the cells in the original monolayer culture.

**Nuclear Changes and Intranuclear Forms of the Virus.** Multinucleated cells occurred often (Fig. 2) in infected cultures. Large and small accumulations of fine aggregates in some of these nuclei appeared to correspond to the intranuclear inclusions seen when similar cells were stained with acridine orange (Fig. 1). Most of the nuclei also showed margination of chromatin. Rupture and disintegration of nuclear membranes and consequent release of nuclear material into the cytoplasm was evident in cells in advanced stages of infection (Fig. 4).

Three morphologically distinct particles were seen in the nuclei of infected cells, i.e., naked particles, enveloped particles, and small, 30–40 m particles. The naked particles were approximately 95–100 m in diameter (Figs. 3, 4). The enveloped particles were seen in nuclear vesicles (Fig. 4) and free in the nucleoplasm (Fig. 7). These particles were 150–170 m in diameter. Those in the nuclear vesicles appeared to obtain the second membrane in the process of budding (Fig. 4). The nucleoids in the enveloped particles were diffuse and stained heavily compared with the compact and lightly stained nucleoids in the naked particles. A third type of particle in the nucleus was approximately 30–40 m in diameter and was of poorly defined structure (Figs. 8a, 8b). It was seen only in association with herpes virus-like particles.

**Changes in the Cytoplasm.** General cytoplasmic degeneration marked by loss of subcellular differentiation, extensive vacuolization, and formation of lysosome-like structures occurred in advanced stages of cellular infection (Fig. 2). As a result of rupture of the nuclear membrane, materials of nuclear origin spilled into the cytoplasm (Fig. 4). Naked and enveloped particles were occasionally seen in the cytoplasm in close association with aggregates of membrane-like structures (Fig. 13).

**Extracellular Virus Particles.** Numerous naked herpes virus-like particles were found in negatively stained preparations of concentrates of growth fluid from infected cultures (Fig. 9). No particles with the outer envelope were seen in such preparations. The nucleocapsid had a morphology similar to that of the herpes virus capsid with hollow-centered capsomeres (Figs. 10, 11). The spatial configuration of the capsomeres was often indistinct, but in some particles (Fig. 11) there appeared to be five capsomeres on an edge, suggesting a total of 162 capsomeres per virion. Negatively stained particles were very similar to intranuclear naked forms of the virus in thin sections (Fig. 12), which rarely had an additional granular coat or "shell" (Fig. 12a).

**DISCUSSION**

We have recently reported the isolation of a cell-associated
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The virus from Marek’s disease infectious blood and presented strong circumstantial evidence implicating this virus in the etiology of the disease (14, 21). The virus was morphologically similar to known herpes-type viruses. Cell culture infection resulted in formation of a syncytial type cytopathic effect with DNA-containing inclusion bodies in the nuclei of many polykaryocytes among groups of cells showing cytopathic effect. The virus appeared to replicate in the nucleus near or within granular aggregates possibly corresponding to the inclusion bodies seen with the light microscope. The naked virus particles were 95—100 nm in diameter, and associated with them in nuclei of some cells were poorly defined particles 30—40 nm in diameter. The nature of the smaller particles and their relation to the virus was not evident, but similar structures have been seen in association with other herpes viruses and have been considered as possible virus genome (13).

Release of the virus from the nucleus appears to occur either by budding through the nuclear membrane or by rupture of the nuclear membrane and escape of the virus along with other nuclear materials into the cytoplasm. The outer envelope appeared to be acquired from the inner nuclear membrane by a process of budding or by budding through membrane-like structures in the cytoplasm.

The process of envelopment of herpes-type viruses as well as the significance of the envelope in infectivity of the virus has been studied by several authors. The origin of the virus envelope has been reported to be from the nuclear membrane (2, 12, 16), plasma membrane, or membrane structures associated with the Golgi apparatus (8, 19). A recent study (7) has clearly demonstrated that the envelopment of herpes viruses occurs predominantly at the nuclear membrane and much less frequently in the cytoplasm. Similar observations are reported here. It is not known whether the membrane-like structures of Fig. 13 were of cytoplasmic origin or were formed by extensive folding and duplication of the nuclear membrane as seen by Dalton and Manaker (6). The role of these structures in envelopment of the virus is not known.

The morphology of the naked virus particles observed in negatively stained preparations was also identical with that of herpes-type viruses. The viral capsid was 95—100 nm in diameter and consisted of 162 capsomers. Enveloped particles, however, were not seen in these negatively stained preparations.

Smith (20) reported the lack of infectivity of naked herpes simplex virus and demonstrated the essential role of the envelope in infectivity of the agent. This has been questioned by Watson et al. (22), who demonstrated that the naked particles were also infectious, though to a lesser degree than enveloped particles. The critical role of the envelope, therefore, has not been confirmed, but the sensitivity of this group of viruses to ether suggests that infectivity may be dependent, at least in part, on the envelope which is lipoprotein in nature. If, however, the naked particles were infectious, the herpes agent would be the only DNA virus with icosahedral symmetry to be sensitive to ether.

The absence of enveloped particles in the growth fluid of infected duck embryo fibroblast cultures reported here, and an earlier observation showing cell-free inoculum from similar cultures to be noninfectious (14), indirectly indicate that the envelop is required for virus infectivity. The demonstration of enveloped particles in the extracellular fluids of Marek’s disease-infected chicken embryo fibroblast cultures which produce extremely low titers of cell-free virus (K. Nazerian, unpublished data) lends further support to this hypothesis. This cell-free virus, however, has been infectious only for cell cultures. Cell-free transmission of Marek’s disease by this virus or otherwise is not yet accomplished.

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Fig. 1. Ultraviolet micrograph of a duck embryo fibroblast culture stained with acridine orange. Distinct inclusion bodies (arrows) are in the nuclei of a polykaryocyte. × 1,000.

Fig. 1. Electron micrograph of a polykaryocyte from an infected culture. Naked virus particles are seen in all the nuclei (arrows). Aggregated masses of nucleoplasm can be seen in most of the nuclei. These may correspond to the inclusion bodies seen with the light microscope. (Fig. 1). The cytoplasm shows extensive granularity and vacuolization. × 8,100.

Fig. 3. Naked herpes-like particles are in the nucleus of this cell. Some particles show a double shell, others have condensed nucleoids, and some contain no nucleoid. Particles of 35–40 mμ in diameter are scattered throughout the nucleoplasm. Such particles are usually seen associated with herpes-type viruses. × 30,000.

Fig. 4. In addition to naked particles in the nucleus, enveloped particles are also seen in two intranuclear vesicles (arrows) and appear to bud into these vesicles. Disintegration of the nuclear membrane can be seen. × 30,000.

Figs. 5, 6. These micrographs demonstrate the budding of the intranuclear particles into the cytoplasm (arrows). The nuclear membrane seems to form the envelope around the virus. × 30,000.

Fig. 7. Both naked (full arrow) and enveloped particles (empty arrow) can be seen in the nucleus of this cell. × 30,000.

Figs. 8a, 8b. These two micrographs taken from two consecutive sections of a cell show three types of particles in the nucleus, naked particles (empty arrows), enveloped particles (white arrows), and aggregates of small 35–40 mμ particles (black arrows). × 30,000.

Figs. 9–11. Negative contrast electron micrographs of particles in the growth fluid of infected cultures. All three figures show typical herpes-like particles. Morphology of hollow-centered capsomeres is more clear in Figs. 10 and 11, while a triangular facet of the capsid (arrows) is shown only in Fig. 11. There appear to be five individual capsomeres on each edge of this triangle. × 126,000.

Fig. 12. Naked forms of the virus in the nucleus of an infected cell. Some particles with or without apparent nucleoid appear to have a granular coat or shell. This additional shell is more clearly seen around one of the particles in inset a. × 69,000.

Fig. 13. Naked (N) and enveloped (E) particles are seen in the cytoplasm of an infected cell. These particles are seen with some aggregates of membrane-like structures (long arrows). Some particles seem to be in the process of envelopment (short arrows). × 69,000.
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