Electron Microscopic Localization of Herpesvirus-type Particles in Marek's Disease

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Upon cultivation of chicken lymphoid tumors induced by Georgia isolate of Marek's disease, clusters of refractile rounded cells appeared consistently in the cell cultures. Intranuclear mature, immature, enveloped, and empty herpesvirus-type particles, usually associated with smaller particles, were observed in all rounded cells in the affected areas. Cytoplasmic inclusions containing mature enveloped virus particles were also seen in the rounded cells. Often fibroblasts growing in the vicinity of the cytopathic effect area contained a few herpesvirus-type particles. The original tumors prior to cultivation did not reveal any virus particles, and the virus in infected cultures was always cell-associated. Control cell cultures neither developed the rounded cells nor demonstrated the presence of any type of virus particles.

Enormous economic losses are sustained annually by the poultry industry in the United States and throughout the world due to Marek's disease (MD; 3, 22). MD is characterized by prominent neoplastic lymphoid lesions in the peripheral nerves of affected chickens. Other organs such as gonad, skin, kidney, spleen, and liver are also frequently involved and show gross and microscopic pathology resembling lymphoid leukosis, a disease complex produced by a group of well-characterized ribonucleic acid containing viruses (30). Although MD appears to be highly contagious under natural and experimental conditions (1, 25), laboratory studies with tissue suspensions have indicated that the etiological agent is usually cell-bound (27). Several attempts to produce MD in susceptible chicks with cell-free inocula have failed (1, 27), and the experimental disease is usually maintained by inoculating potent cellular preparations into young chicks. The explanation that the laboratory disease is a result of cell transplantation has been ruled out on the basis of chromosome studies (19). Recently, herpesvirus-type particles have been reported in cell cultures infected with JM strain of classical MD (16) and HPRS 16 strain of acute MD (5). Although these experiments suggest the presence of a viral genome in MD tumors *in vivo*, attempts to observe virus particles in the electron microscope in such tumors have failed (16).

We studied the cultural and virological aspects of lymphoid tumors in chicks induced by one of the agents of MD, the GA strain. This report describes the alterations in cell cultures derived from MD tumors, the relationship between the

presence of herpesvirus-type particles and occurrence of cytopathic effects in cell cultures, and the electron microscopic histology of the kidney tumor fragments from which the cell monolayers originated.

MATERIALS AND METHODS

MD agent. The Georgia (GA) isolate of MD was kindly supplied by S. C. Schmittle of the Poultry Disease Center, University of Georgia, Athens, Ga. Visceral lymphoid tumors developed within 4 weeks following intra-abdominal or subcutaneous inoculation of newly hatched Athens-Canadian chicks with GA-infected blood or liver tumor cell suspension. The tumor material for this study was obtained from 4-week-old chicks showing massive tumors in the ovary, liver, spleen, and kidney. Skin lesions were also evident in some of the affected chicks. Pieces of tumors were removed and immediately fixed for histopathology and electron microscopy. The remaining portions of the same tumors were used for cell culture studies.

Cell culture. The chick kidney tumors induced by GA isolate were minced, trypsinized in three cycles, washed, and suspended in growth medium to a final count of 1.5×10^6 to 2.0×10^6 cells per ml. To each 60-mm Falcon dish or 30-ml flask, 5 ml of the cell suspension was added, and the cultures were incubated at 38 C in a humidified atmosphere containing 3.5% CO₂. Fragments of the tumor which resisted trypsinization were plated without a count. The growth medium consisted of Eagle's minimal essential medium (MEM) with Hanks' balanced salt solution (HBSS), 80%; calf serum (Grand Island Biological Co., Grand Island, N.Y.), 10%; tryptose phosphate broth, 10%; and sodium bicarbonate, 0.095%. Penicillin and streptomycin, 200 units and 200 µg, respectively, per ml were added. The normal chick kidney cell

cultures were prepared as above. An inoculum of 0.1 to 1.0 ml of whole blood or 10% liver and kidney cell suspensions was added to chick kidney cell cultures. After 24 to 48 hr, the cultures were washed with HBSS and fresh growth medium was added. The secondary cultures were prepared by trypsinizing overgrown primary cell monolayers, and then the cells were plated in fresh growth medium. The maintenance medium was the same as growth medium except that it contained 2% calf serum.

Cell culture monolayers for electron microscopy. Infected cell monolayer cultures selected by light microscopy for various degrees of cytopathic effect (CPE) were fixed *in situ* by a flat-face embedding method (2), modified by J. H. Monroe (*personal communication*). The cell sheet was washed three times with HBSS, pH 7.3, and once with 2.5% glutaraldehyde in phosphate-buffered saline (PBS); then 4 ml of glutaraldehyde solution was added to each cell monolayer. After 1 hour of fixation at room temperature, the monolayer was rinsed twice with PBS and postfixed in Dalton's chrome-osmium (6) for 30 to 60 min at room temperature. The cell sheet was then rapidly rinsed with distilled water several times. Dehydration was in ethyl alcohol up to 70%, followed by 90, 95, and 97% hydroxypropyl methacrylate (HPMA) for 15 min each. The 97% HPMA contained 0.1% Toluidine Blue. The cell sheet was then processed through progressively increasing concentrations of Epon-embedding mixture in 97% HPMA over a period of 1 hr, and embedded in Epon alone according to Luft (12). The embedded cell sheet can be peeled off from the Falcon plastic container and is then ready for area selection by light microscopy. The cells are stained blue and can be easily identified. The stain does not interfere with the fine structure at the electron microscope level. After photography, the areas of interest were marked with a slide marker (C. W. French, Weston, Mass.), replacing a regular objective in the microscope turret. The marked areas were cut out with a cork borer and fastened with epoxy glue to a cylindrical block fitting the microtome chuck. The monolayers were sectioned in a plane parallel to their substrate. For electron microscopy of large fields at low magnification (about 200 times), ribbons of sections were picked up on Formvar-carbon covered "M and M" grids with a 1-mm-diameter circular opening (no. 2509, E. F. Fullam, Inc., Schenectady, N.Y.). An additional carbon film was evaporated onto the sections for stability under the electron beam.

Millipore membranes for electron microscopy. Cell culture fluids were processed through 1.2- μ m membrane filters (Millipore Corp, Bedford, Mass.), pretreated with calf serum. The membranes were then processed according to the method of McCombs et al. (14), and sectioned in a plane perpendicular to the plane of the membrane.

Whole tissue and cell culture pellets for electron microscopy. Cell cultures were washed with HBSS, scraped off the plastic surface with a rubber policeman, and centrifuged gently into a pellet. The cell pellets and the small pieces of original tumors were fixed in 2.5% glutaraldehyde in PBS, postfixed in

Dalton's chrome-osmium, stained with 2% uranyl acetate in 50% ethyl alcohol, and after dehydration in graded alcohols and propylene oxide, they were embedded in Epon (12). All sections were made with an LKB microtome equipped with a diamond knife, stained with lead citrate according to Venable and Coggeshall (29), and studied in a Siemens Elmiskop I.

RESULTS

Cell culture alterations. A series of 17 cultures was prepared from chick kidney tumors induced by GA isolate of MD. Within 48 hr, individual cell colonies could be seen under the microscope. The fragments of tumors which resisted the trypsinization adhered to the plastic surface, and proliferative fibroblast-like cells originated from them. A few small discrete foci of rounded cells (CPE) appeared on the fourth day in 12 out of 17 cultures. The number and size of the CPE areas grew with subsequent incubation. By the seventh day, all 17 cultures were positive for CPE that consisted of clusters of round and refractile cells which had the tendency of piling on top of each other (Fig. 1). The altered rounded cells were highly basophilic in nature, and their structure was not clearly revealed with Wright's or May-Grunwald-Giemsa stains. These rounded cells were attached firmly to the monolayer in the initial stages (Fig. 2), and the "sloughing off" phenomenon was not noticed until the cultures were 18 to 20 days old. At this time, cellular debris was evident in the culture fluid. Time-lapse photography of the CPE areas showed that the growing fibroblasts were continuously being altered into rounded cells. This process appeared to be a self-destructive one, since the altered rounded cells (after going through several phases) were lysed. Mitosis among these cells was not observed (*unpublished data*). When cell monolayers that contained CPE were subcultured, with or without the addition of fresh normal chick kidney cells, the CPE was observed on three subsequent passages.

A similar CPE was observed when normal chick kidney cell monolayers were infected with cellular suspensions of chick kidney and liver tumors. However, the development of the CPE was delayed to the ninth day, and a more degenerative response was found in the CPE area than was evident in the case of direct kidney tumor culture. Similar lytic response was also observed in areas in which rounded cells were not present, as well as in control cultures inoculated with high doses of normal blood and kidney and liver cell suspensions, suggesting a toxic response to cellular inoculum and cell debris.

Cell fluid from 10 cell cultures showing 30 to 50 CPE areas per culture was collected and passed

FIG. 1. Typical clusters of unstained rounded cells (CPE) observed on the 6th day of direct culture of GA chick kidney tumor. After 18 hr of further incubation, the whole cell sheet was processed for electron microscopy. $\times 210$.

FIG. 2. Same area as in Fig. 1 after *in situ* fixation, staining, and embedding for electron microscopy. Note little or no change in the morphology of the clumps occurred during these procedures. $\times 210$.

through a 1.2- μ m membrane filter (Millipore Corp.). The filtrate was inoculated in normal chick kidney cell cultures. No cellular alterations of any kind were observed. The filter membrane itself was processed for electron microscopic examination. Herpesvirus-type particles were found in the cellular debris collected on the filter membrane during filtration.

Herpesvirus-type particles. The identity of the virus-bearing cells is demonstrated by a comparison of Fig. 3 and 4. The light micrograph in Fig. 3 was made before sectioning, and the electron micrograph in Fig. 4 after sectioning. Some cells in Fig. 3 do not appear in Fig. 4 because of the thickness difference between the whole clump (probably over 100 μ m) and the microtome section (approximately 0.1 μ m). All 27 cells in Fig. 4 have nuclear and cytoplasmic herpesvirus-type particles. A representative cell (no. 25) is illustrated in Fig. 5. Herpesvirus-type particles (capsid diameter approximately 100 nm) were randomly distributed throughout the three main regions of such cells: (i) the nucleus, (ii) cytoplasm, and (iii) cytoplasmic inclusions.

The nucleus exhibits a mixture of empty, immature, and mature virus particles, with the latter being in noticeable minority (Fig. 7). Also

present within nuclei are mature capsids surrounded by double envelopes (Fig. 9). Another consistent component of the nuclei of rounded cells is a population of small particles (approximately 40 nm in diameter) randomly scattered throughout the nucleoplasm (Fig. 10). Such particles are not bound by membranes, and a small proportion of them appears to have empty cores. The nuclear chromatin is sparse and very margined. A few patches of coarse granular material are randomly scattered. The nuclear envelope appears normal.

Within the cytoplasm, in addition to normal smooth and rough endoplasmic reticulum, mitochondria, empty vesicles, and a few nonspecific bodies often seen in cultured cells, there are a few randomly scattered mature virus particles without envelopes (Fig. 5a).

The third region is a large and prominent membrane-bound cytoplasmic inclusion containing a fine granular matrix (Fig. 5, 6). Within this matrix are found mature and almost exclusively enveloped virus particles (Fig. 6). The space between the capsid (C) and the envelope (E) contains amorphous material which is preferentially in intimate contact with the envelope rather than with the capsid. In sections of some other

FIG. 3. *Clump II* of Fig. 2 rotated clockwise 90° in the plane of the page. Cells are numbered for orientation purposes. Approximately $\times 900$.

FIG. 4. Electron micrograph of cells in *clump II*. Corresponding images of the same cells bear identical numbers as in Fig. 3. Details of cells 22 and 25 are illustrated in Fig. 5, 6, 7, and 8. Approximately $\times 1,000$.

FIG. 5. Overall appearance of cell 25 in clump II. The areas indicated by large arrows in inclusion (IN) and nucleus (NU) are further magnified in Fig. 6 and 7. The small arrows point to cytoplasmic virus particles. Approximately $\times 9,700$. (a) Mature unenveloped cytoplasmic virus particles. CY, cytoplasm. Approximately $\times 67,800$.

FIG. 6. Detail of peripheral area of inclusion (IN) in Fig. 5, showing the appearance and most frequent location of enveloped herpesvirus-type particles. Approximately $\times 67,800$.

FIG. 7. Detail of area at arrow in nucleus of cell 25, Fig. 5. Randomly scattered empty (e), immature (i), and mature (m) herpesvirus-type particles are illustrated. Approximately $\times 67,800$.

FIG. 8. Detail of cytoplasmic inclusion (IN) in cell 22 of Fig. 4. Numerous small vesicles (SV) aggregated in chains or clusters are illustrated. CY, cytoplasm. Approximately $\times 67,800$.

FIG. 9. Enveloped herpesvirus-type particles seen within nuclei. The viral capsid (C) is surrounded by two envelopes, E_1 and E_2 . Approximately $\times 67,800$.

FIG. 10. Detail of nucleus in a cell within clump II (Fig. 4). Illustrates the appearance of small nuclear particles. A few with empty cores are seen at arrow. Approximately $\times 67,800$.

FIG. 11. Necrotic cell with virus particles in nuclear (a) and cytoplasmic (b and c) areas. The plasma membrane is lysed but the cellular contents remain clumped. Virus particles in areas a, b, and c are illustrated in Fig. 11a, 11b, and 11c, respectively. Fig. 11, $\times 6,500$; Fig. 11a, $\times 34,000$; Fig. 11b and 11c, $\times 66,100$.

cells, more than one such inclusion is occasionally seen, and a few of them (cells 7 and 8, Fig. 4) appear to have lost their granular matrix. In addition to enveloped virus particles, these inclusions contain a population of characteristic small membrane-bound vesicles (about 40 nm in diameter). These vesicles aggregate in chains or clusters in the proximity of the limiting membrane within the inclusion (Fig. 8). The area surrounding the inclusion consistently shows a multitude of irregular bodies filled with a material resembling the matrix of the inclusion (upper-left corner, Fig. 6). Occasionally enveloped virus particles are seen outside, but always in the immediate proximity of the inclusion. These are usually embedded in inclusion matrix-type material. Occasionally, the envelopes of virus particles within the inclusions are only partially seen as in Fig. 8.

Necrotic cells and cellular debris within clusters of rounded cells were observed. Some herpes-virus-type particles were occasionally seen within nuclear and cytoplasmic fragments (Fig. 11a, b, c). Single, clearly extracellular virus particles distinctly away from cellular debris were never seen.

Another type of virus-containing cytoplasmic inclusion is illustrated in Fig. 12 and 13. Some inclusions appear to consist of phagocytized coarse granular and membranous cellular debris. They contained unenveloped virus particles of the empty, immature, and mature varieties, and were seen only in fibroblast-like cells in the immediate vicinity of CPE areas. The nuclei of cells with such inclusions did not show any virus particles. A few scattered intranuclear herpes-virus-type particles were observed in fibroblasts growing in the neighborhood of CPE (nucleus N3, Fig. 12 and 14).

Electron microscopical histology. The fragment of a kidney tumor, from which the cell monolayer grew and virus-containing CPE areas appeared (Fig. 15), was examined under the electron microscope. Figures 16 and 17 illustrate the histology of such a fragment. The central portion of the area marked "X" in Fig. 15 consisted mainly of cellular debris with only a few scattered cells remaining (Fig. 16). The peripheral portion of the fragment (X in Fig. 15) is illustrated in Fig. 17. Four differentiated areas, bearing no resemblance to the original kidney tissue, may be recognized: an inner necrotic area (X), a multilayered area of squamous epithelial-like cells, an outer layer of cuboid epithelial-like cells, and finally a projection of cells which grows into fibroblast-like cells (F). Surprisingly, no virus was seen in any of these areas. Figure 18 illustrates the distribution of virus-containing cells in the outlined area of

Fig. 15. There is no obvious resemblance between the light and electron micrographs of this area because the thin section was made at the level of the fibroblasts in the monolayer, rather than through the piled up rounded cells as in Fig. 3 and 4. Each one of the labeled cells was studied at higher magnifications to determine the presence or absence of virus particles. Cells labeled P and N are positive and negative, respectively, for virus particles. The clustering of P cells is emphasized by the dashed lines. Some positive rounded cells are seen in the upper part of the figure. These are surrounded by some positive and negative fibroblasts that also appear along the left margin of the section. Only a few positive fibroblasts are scattered among the multitude of negative cells occupying the majority of the illustrated area. The positive fibroblasts in the right-central part of the figure were neighboring with rounded cells that remained outside the plane of this section. No fibroblasts away from the CPE areas were ever seen to contain virus particles.

A representative portion of the original kidney tumor tissue, from which the monolayer in Fig. 1 grew, is illustrated in Fig. 19. It is highly invaded with typical lymphoid-type cells. Only a few aberrant elements of parenchymal tissue remained. A part of a proximal convoluted tubule with its lamellar brush border and associated basement membrane is illustrated on the upper-right side. Figure 20 demonstrates the nuclear projections often observed in the lymphoid-type cells. Similar lymphoid-type cells were also seen to invade the spleen, liver, nerve, and ovary tumor tissues obtained from the same chick. No virus of any type was found in these specimens prior to cell cultivation. The search to localize virus particles in the original MD tumor tissues obtained from other chicks included the following number of specimens: bone marrow, 1; spleen, 2; nerve, 2; ovary, 3; kidney, 3; buffy coat, 3; respiratory epithelium, 1. Each of these tissues has been intensively searched in consecutive sections of various areas without success.

DISCUSSION

In vitro techniques of growing and maintaining the agent of MD are in a developmental stage and hopefully quantitative methods will eventually evolve, which obviously will have an advantage over the time-consuming and expensive in vivo testing. Appearance of altered, rounded cells (CPE) in GA-infected cell cultures seems to be highly specific and easily identifiable. Our findings are in complete accord with the reports by Churchill and Biggs (5) and Solomon et al. (27), who also showed the specificity of the appearance

FIG. 12. Cells within rectangular area enclosed by solid line in Fig. 18. Illustrates appearance and location of virus-containing phagocytic-type cytoplasmic inclusions (IN-1 and IN-2). Nuclei N-1 and N-2 of cells bearing such inclusions appeared free of virus. Approximately $\times 4,940$.

FIG. 13. Detail of inclusion IN-2 in Fig. 12. All virus particles within such inclusion are unenveloped. Approximately $\times 49,100$.

FIG. 14. Illustrates presence of herpesvirus-type particles in nucleus N-3 of fibroblast-like cell in Fig. 12. Approximately $\times 75,000$.

FIG. 15. Light microscope photograph of an area in cell culture monolayer. Tumor fragment is marked "X." Electron micrographs were made within the fragment (Fig. 16), at the periphery of the fragment (Fig. 17), and in the area within the rectangular outline (Fig. 18). Approximately $\times 168$.

FIG. 16. Detail of central portion of tumor fragment "X" in Fig. 15. The arrows indicate the few remaining whole cells. Approximately $\times 3,640$.

FIG. 17. Detail of peripheral portion of tumor fragment "X" in Fig. 15. Approximately $\times 3,640$.

FIG. 18. Composite electron micrograph of the area outlined by solid line in Fig. 15. Illustrates fibroblast-like cells in the neighborhood of rounded cells. P = positive for virus, N = negative for virus. Approximately $\times 550$.

FIG. 19. Illustrates the appearance of typical infiltrating lymphoid-type cells in the kidney tumor tissue used to initiate the culture monolayer illustrated in Fig. 1. Approximately $\times 3,500$.

FIG. 20. Detail of nuclear projections in lymphoid-type cells in Fig. 19. The arrow points to a finger-like nuclear projection seen in cross section. Approximately $\times 21,700$.

of CPE with two other isolates of MD and produced the disease in susceptible chicks with cell cultures containing CPE.

The presence of herpesvirus-type particles in cell culture pellets infected with two strains of MD (HPRS 16 and JM) has been previously described (5, 16). However, the virus-containing cells have not been identified, and the mode of virus release remains unknown. This investigation establishes that herpesvirus-type particles (100 nm) and small intranuclear particles (40 nm) are consistently found in the rounded cells (CPE), and that a few of the fibroblasts growing in close proximity to CPE also contain the virus. The techniques of *in situ* fixation and flat-face embedding and sectioning have proven to be the valuable tools in establishing the identity of virus-carrying cells. Several morphological varieties of herpesvirus-type particles were located in cells infected with GA strain of MD, namely (i) immature with doughnut nucleoid, (ii) mature with dense nucleoid, (iii) empty, and (iv) enveloped. Although enveloped virus particles were observed predominantly in the cytoplasmic inclusion and only rarely in the nucleus, the mechanism of envelopment and a frequent site of envelope formation could not be identified. Enveloped herpesvirus-type particles in cell cultures infected with JM and HPRS 16 strains of MD were not mentioned by other investigators, but small nuclear particles similar to those reported in this study have been noted also by Churchill and Biggs (5) in MD infected cultures. Morgan et al. (15) also found them in cultures infected with herpes simplex virus, as did Rabin and Jenson (20) in a study of herpes simplex hepatitis in mice. Although the morphology of herpes simplex virus is dependent on cultural conditions (4), we cannot yet provide evidence whether the small particles are components of herpesvirus or are an unrelated material.

The nature of the cytoplasmic inclusions (Fig. 5) in the altered rounded cells also remains to be elucidated. They have not been previously reported in MD cell cultures, but cytoplasmic inclusions containing herpesvirus-type particles have been described in the Lucké renal adenocarcinoma in frogs (13) and in cytomegalovirus-infected mouse liver and cell cultures (23, 24). Their role in the production of virus particles in our system has not been identified, but enveloped virus particles were frequently seen in these inclusions. Since no extracellular enveloped virus particles were seen and since it appears that the enveloped virus particle represents the infectious moiety (17, 26, 28), these inclusions may be trapping the infectious particles and thereby reducing

the spread of the infection throughout the monolayer.

The other type of cytoplasmic inclusions (Fig. 12) was located in fibroblast-like cells growing near rounded or degenerating cells. These inclusions contained herpesvirus-type particles and cellular debris. Phagocytosis of the cell debris, containing the virus or viral genome, by new cells may represent one of the modes by which the infection spreads in the cell culture.

Electron microscopic and cultural studies strongly suggest that the CPE-inducing agent is closely associated with cells. Cell-free fluids of infected cultures failed to produce CPE in the fresh cultures. Similarly, an extensive search for free virus particles in cell cultures was fruitless. The virus particles were always found to be intracellular or associated with cell debris. Such close cell association confirms the earlier observations on this subject (5, 27).

It has been shown that infected cell cultures which do not develop CPE do not produce MD in chicks (27). Since only CPE-containing cell cultures are likely to produce MD, and since our findings show that each and every rounded cell in the CPE area contains herpesvirus-type particles, it would strongly suggest that the genome of these virus particles may be involved in the induction of MD. However, extensive experimentation may be necessary to establish the herpesvirus-type particles as the sole etiological agent of MD.

The herpesvirus-type particles have also been observed in Burkitt's lymphoma cultures (7, 10, 18), frog kidney tumors (9, 13), and in certain human leukemias (11). Although the oncogenicity of any of the herpesvirus-type particles has not been unequivocally established, their persistent presence in several neoplastic diseases has stimulated a worldwide research effort commensurate with the importance of the problem.

Our failure to observe virus particles in original MD tumors and the tumor fragments from which the virus-containing cell monolayer originated deserves comment. The electron microscopic search for virus particles in the original tumors was exhaustive, but it is probable that they would be missed if the virus content was very low. Similar examples have been reported in the literature with Burkitt's lymphoma (8, 21) and other neoplastic conditions in man (11) in which herpesvirus-type particles were observed only after making cell cultures from the original tumors. One possible explanation is that the synthesis of herpesvirus-type particles, observed in MD cell cultures, is repressed in the tumor tissue due to unknown inhibition mechanisms that are par-

tially or completely removed during *in vitro* cultivation, allowing the formation of virus particles.

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