Ultrastructural and Biological Properties of a Cytomegalovirus Rescued from a Human Paraganglioma

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SUMMARY

In a tissue culture isolate from a biopsy of a recurrent paraganglioma, lymphoblast-like cells were found growing in suspension from the first culture on. The suspension culture has now been growing continuously for 14 months. These cells occasionally contained cytoplasmic areas of anastomosing tubular structures showing continuity with endoplasmic reticulum. Cell-to-cell contact between these cells and WI-38 cells resulted in the appearance of a virus in the latter. This virus possesses the ultrastructural, biophysical, and immunological properties of a cytomegalovirus. The patient’s serum contained antibodies against cytomegalovirus but not against herpes hominis I and II or against Epstein-Barr virus. The evidence suggests that the presence of cytomegalovirus in lymphoid cells may stimulate continuous growth in tissue culture.

INTRODUCTION

A continuing study that involves the electron microscopic analysis of a series of tissue culture isolates from human solid tumors has been concerned essentially with determining the presence or absence of virus in these isolates, primarily by electron microscopic monitoring. The present report describes the demonstration of CMV in 1 of the tissue culture cell lines developed in this study.

MATERIALS AND METHODS

Materials. The material for the original tissue culture isolate was obtained from a biopsy of a recurrent paraganglioma (originating near the organ of Zuckerkandl) in an 81-year-old woman. The original biopsy material was finely minced and either cultured directly in T-60 flasks or trypsinized before seeding. The trypsinized material failed to grow either as a monolayer or as a suspended culture. The minced material placed directly in flasks grew in a medium consisting of 20% inactivated PCS (Flow Laboratories, Inc.), BME with Hanks’ salts, vitamins, essential amino acids (Grand Island Biological Company), glutamine, and 100 units penicillin-streptomycin (Media Unit, NIH). For subcultivation of WI-38 cells, 0.25% trypsin was used. Then the medium containing floating cells were decanted and replaced by fresh medium. Thereafter, supernatant fluids of the infected WI-38 cell strain were used for further infection of WI-38 cell cultures. WI-38 cell cultures served as controls, being sham infected with supernatant fluids of control cultures of strain WI-38.

Electron Microscopy. Floating cells from Culture 13 were examined at different times by light and electron microscopy and were checked with fluorescent antibody techniques. Samples were taken 2, 4, 6, and 8 dpi.

Light Microscopy. Monolayers of control and infected WI-38 cells grown on coverslips in Leighton tubes were fixed with Carnoy’s fluid after 3 rinses with phosphate-buffered saline and stained with May-Grünwald-Giemsa stain.

Electron Microscopy. Floating cells from Culture 13 were centrifuged at 1200 rpm (PR International Centrifuge), and the cell pellets were fixed with 3% glutaraldehyde. Monolayers of WI-38 cells, controls and infected cells, were fixed in situ with 3% glutaraldehyde (16). Postfixation was carried out with chrome-osmium (4). Dehydration and embedding in Epon-Araldite (12) was done according to standard methods. Sections were cut with an LKB Ultrotome, double stained, and examined in a Siemens 1A Elmiskop with a 50-μm objective aperture and an accelerating voltage of 80 kV.

A virus pool was prepared by 1-time freezing and thawing of the infected WI-38 cells. A portion of the supernatant fluid was frozen with an equal amount of 70% sorbitol; the other part was frozen without any addition (1). The pools were titered in WI-38 cells, and physicochemical and immunological characterizations were done (14).

Thermal Inactivation. The undiluted virus pool and a 1:10 dilution were heated at 56°C for 30 min to inactivate the virus.

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1 Material obtained under Contract NIH-69-2074 with St. Joseph’s Hospital, Tampa, Fla.

2 The abbreviations used are: CMV, cytomegalovirus; PCS, fetal calf serum; BME, basal medium Eagle’s; dpi, days postinfection; CPE, cytopathogenic effects; HSV, herpes simplex virus; FA, fluorescent antibody; EB, Epstein-Barr; CF, complement fixation.
CMV in Human Cultures

Tissue Culture 13

...inactivated FCS were treated at 56° and 37°, respectively, for 30 min in a water bath. These samples were inoculated onto WI-38 cells and were observed for CPE for 15 days.

pH Effect. A 1:10 dilution of virus pool in 2% FCS BME (Hanks') adjusted to pH 2.95 was incubated for 30 min at 37°. A control prepared in BME (Hanks') with a pH of 7.15 was also incubated. The samples were inoculated onto WI-38 cells and were observed for CPE as in the previous experiment.

Ether Sensitivity. An equal amount of undiluted virus pool and ether was mixed and incubated at 4° overnight. After the ether was evaporated, the treated virus pool was diluted 1:10 in 2% FCS BME (Hanks'). The control and the treated material were inoculated onto WI-38 cells.

Cell Infectivity. To rule out that this isolate might be a member of Herpesvirus Group A (11), we inoculated the virus pool diluted 1:10, onto primary rabbit kidney, baby hamster kidney, and Cercopithecus monkey kidney cells. Parallel studies were performed with a known CMV (AD-169) and HSV Type I (JW) control virus. These cultures then were observed for CPE for 10 days.

Neutralization. Two dilutions of the virus (10⁻².0 and 10⁻³.0) were used in the neutralization test with rabbit anti-CMV (prepared against strain AD-169), guinea pig anti-HSV (Type I), and the patient's own serum. A 1:16 dilution of anti-CMV and anti-HSV and a 1:8 dilution of the patient's serum were used in this test. The test procedures were described previously (1).

FA Technique. In the direct FA test, 2 sera, a human CMV-positive and a hyperimmune serum produced in rabbits (10) against acetone-fixed, CMV-infected cells of strain AD-169 were used. In addition, a serum against an EB virus (the clone of the Burkitt's lymphoma P3J) was examined.

In the indirect FA test, a serum against HSV type I (JW strain) and a human serum containing antibodies against HSV were used.

CF. The CF antigen was prepared against the isolate from infected WI-38 cells and floating cells from Tumor Culture 13. The CMV antigen against AD-169 was tested by microtiter with the use of 2 units of complement (17). These antigens were tested against patients' serum and a human CMV-positive serum.

RESULTS

Original Tumor

Light microscopic examination of the original tumor tissue revealed a typical malignant paraganglioma with diffuse infiltration of lymphocytes and a few circumscribed foci of well-organized lymphoid follicles.

Tissue Culture 13

The original tissue culture isolate (Culture 13), started August 11, 1969, grew as a mixed culture consisting of cells adhering to the glass and forming a monolayer and a few others growing in suspension. After 9 months of cultivation, the cultures were mainly composed of floating cells. These cells have the appearance of immature lymphoid cells and are rather uniform in size and morphology. Only a small percentage of the cells is dead and appears as "ghost-like" cell remnants among what appear to be lymphoblasts (Fig. 1).

The cytoplasm is poor in rough endoplasmic reticulum but contains numerous free polysomes; a large Golgi area; frequent lipid bodies; and mitochondria, some of considerable size (Fig. 1, M).

In less than 10% of the sectioned cells, cytoplasmic inclusion bodies as illustrated in Fig. 2 are found (see also Fig. 1, arrow). These bodies are composed of electron-dense tubular structures with an average diameter of 20 μ. These structures are in close association and, in some instances, in direct continuity with the rough endoplasmic reticulum (Fig. 2, inset). Virus particles were not observed in cells of line 13.

Infected Cells of Strain WI-38

Light Microscopy. The controls have the characteristic appearance of fibroblast-like cells (Fig. 3). The 1st changes, 2 days after infection, are (a) a stronger affinity to the stain, (b) the rounding of many cells (Fig. 4), and (c) the appearance of small inclusions in the nuclei (Fig. 5, arrows). Two to 3 days later (4 dpi), the nuclear inclusions are prominent and replace most of the nuclear matrix. At a later time (6 to 8 dpi), round, droplet-like inclusions appear in the cytoplasm, usually adjacent to the cytocentrum (Fig. 6, arrow), and the development of a syncytium is frequent, resulting in giant cells containing up to 8 or 10 nuclei (Fig. 6).

Electron Microscopy. Control WI-38 cells are clearly different from the lymphoid cells (Fig. 1), being elongate and possessing well-differentiated ergastoplasm oriented along the long axis of the cell (Fig. 7, inset). Ultrastructural changes following infection with the isolate of Tissue Culture 13 parallel the light microscopic observations. The 1st changes (2 dpi) are observed in the nuclei of the infected cells (Fig. 7). They are manifested by the appearance of viral precursors embedded in an electron-dense, fibrillar viral matrix.

Some viral precursors in the nucleus are empty shells with 1 membrane, some are double shells (with 2 membranes) (Figs. 8 and 9), and some particles contain electron-dense centers surrounded by 1 membrane (Fig. 9).

As seen in Figs. 8 and 9, in some areas the viral matrix condensed in the nuclear inclusion body is made up of fibrillar material. Sometimes the nuclei also contain electron-dense material, probably of viral origin, arranged in a skein-like form. This material is often surrounded by empty particles (Fig. 8) and is distinct from the viral nuclear inclusion body. No changes could be detected in the nucleoli. They appear to be completely separated from both kinds of viral nuclear inclusion bodies.

In the cytoplasm, the ergastoplasm becomes disorganized (Fig. 7). At this stage, virus particles are seen infrequently in the cytoplasm and in extracellular spaces.
Antigens were prepared from uninfected and infected cells with 10^6 cells/ml.

<table>
<thead>
<tr>
<th>Serum</th>
<th>CMV AD-169 (1:8)</th>
<th>Isolate propagated in WI-38 cells (undiluted)</th>
<th>Suspended lymphocyte (undiluted)</th>
<th>WI-38 cells (1:8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient’s CMV-positive</td>
<td>1:32</td>
<td>1:32</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>CMV-positive</td>
<td>1:8</td>
<td>1:32</td>
<td>Negative</td>
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Four to 8 days after infection, the nuclear inclusion bodies enlarge, and incomplete virus particles can be found in the perinuclear space (Fig. 10, arrow). Virus particles were observed budding from the cytoplasmic matrix into vacuoles (Fig. 10, inset), adding a further envelope to their structure. Infrequently, budding was observed at the cellular membrane.

Particles accumulate in the cytocentrum (Golgi area) (Fig. 11, arrows), mostly singly, surrounded by a vacuole. Later, lysosomal bodies appear in this area (Fig. 12). Vacuoles containing virus particles and lysosomes appear to fuse (Fig. 12, inset).

**Biological Properties of the Isolate.** The virus was completely inactivated at 56° after a 0.5-hr incubation. The sample kept at 37° for the same period produced typical CMV-type CPE.

No CPE were observed in different dilutions of the virus of pH 3.0-treated samples; however, the control samples at pH 7.15 titrated 10^6.0. The results suggest that like other herpesviruses, the isolate is acid labile.

The virus was sensitive to ether treatment. The undiluted ether-treated inoculum produced 1 to 2 foci of rounded cells. However, the untreated controls showed a titer of 10^6.0.

Cell infectivity studies indicated that the virus did not induce CPE in any of the cell lines tested, however, CPE 48 hr postinfection were observed with HSV. These results rule out the possibility that the isolate belongs to Herpes Group A, since only HSV I and II infect the variety of cells studied.

A nonspecific staining was observed in the acetone-fixed lymphocytic cell cultures with antihuman conjugated globulin. In the indirect test, WI-38 infected cells (3 dpi) exhibited also a nonspecific staining with human anti-HSV serum, but experimentally produced serum did not stain the cells.

In direct tests, early infected cells also showed some nonspecific staining in normal WI-38 cells with human CMV serum. However, some infected acetone-fixed cells showed nuclear staining with this serum. In the previous studies, this serum was found to contain no neutralization titer against HSV Type I and varicella-zoster virus and also had no FA staining on a clone of the Burkitt’s lymphoma (P3J) HR1K cells when used at 1:10 dilution.

Cells infected for 6 and 8 days treated with rabbit anti-CMV conjugated serum stained both nuclear and cytoplasmic inclusions. The 1:8 and 1:16 dilutions of the serum stained 70% of the WI-38 cells 6 dpi. The ratio of nuclear to cytoplasmic inclusions stained was 10:1. Ninety % of the cells infected for 8 days had nuclear staining, and 30% of them showed, in addition, staining of cytoplasmic inclusions. Both of these serum dilutions were negative when tested on normal WI-38 cells. The FA results indicated that the isolate is a CMV and may be related to strain AD-169, since the experimental antiserum showing FA reaction was produced against strain AD 169.

**DISCUSSION**

Electron microscopic analysis clearly indicates that this virus belongs in the herpes group, first, because it replicates primarily in the nucleus and, second, because of the 3 characteristic types of particles, 1 with a single shell, 1 with a double shell, and 1 with an electron-dense nucleoid surrounded by a single shell. Ultrastructural detail of the nuclear inclusion bodies is typical of the inclusion bodies characteristic of CMV infection; large areas of amorphous, electron-dense material interspersed by less electron-dense areas containing the 3 types of naked herpes-type particles. Likewise, the close association of lysosomal bodies with the virus particles in the cytoplasm is a characteristic feature of CMV-infected cells (18).

The demonstration of the presence of subclinical or carrier-state CMV infection in patients with neoplastic disease is obviously not new (5, 6). Also, as indicated by Smith (18), "The occurrence of the infection of the salivary glands with cytomegalovirus" as a chronic process with prolonged excretion of the virus in the saliva and urine, together with the prevalence of the disease in young children, provides an explanation for superimposed infections in pertussis, other chronic lung diseases and debilitating conditions." What is worthy of note is, first, the presence of immature lymphoid cells growing in suspension in a paraganglioma isolate during 9 months in serial culture and, second, the fact that it was possible to rescue CMV from these cells by cell-to-cell contact.

The facts of the continuous growth of lymphoid cells in this isolate and that the serum of the patient contained antibody against CMV but not against HSV Types I and II or EB virus are perhaps related to the fact that CMV infection induces mononucleosis in previously healthy individuals (9). The evidence thus suggests that CMV, as well as EB virus (7, 8, 15), may stimulate lymphoid cells to grow continuously in suspension culture.

An interesting factor is the presence of cytoplasmic inclusions of a reticular array in these stimulated lymphoid cells. Electron-dense structures of similar morphology have been found repeatedly. [Note added in proof: Recently similar structures have been found in human tumors of mesenchymal origin (J. G. Sinkovics, personal communication.)] not only in virus-infected cells (13) but also in cells of different pathological disorders apparently free of viral infections (2, 3).
It was pointed out recently that due to the latter fact the viral origin of these cytoplasmic inclusions might be questionable (3). Contrarily, the rescue of a CMV from lymphoid cells of a newly established cell line containing inclusions of such a kind suggests the possibility that, at least under certain circumstances, these inclusions may represent a characteristic marker for viral infections.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Lymphoid blast cells from Tissue Culture 13 after 9 months of cultivation. Cytoplasmic inclusion bodies are present in a few cells (arrows). Mitochondria (M) are large and swollen. X 5,300.

Fig. 2. Inclusion body composed of electron-dense anastomosing and branching tubular structures, which have an average diameter of 20 m. The inset reveals the close association of these structures with the rough endoplasmic reticulum (arrows). X 55,000. Inset, X 70,000.

Fig. 3. WI-38 cells. Control cells with the appearance of fibroblasts. X 125.

Fig. 4. WI-38 cells 2 days after infection with the new isolate. Many cells are rounded and stain intensely. X 125.

Fig. 5. WI-38 cells 2 days after infection with the new isolate. Some of the nuclei contain small inclusion bodies (arrows). X 250.

Fig. 6. Six days after infection, nuclear inclusions are prominent, and droplet-like inclusions are present in the cytoplasm (arrows). Giant cells containing 8 to 10 nuclei are frequently found at this stage of infection. X 250.

Fig. 7. WI-38 cell infected with the new isolate. The nucleus contains a large inclusion body with numerous incomplete virus particles. The cytoplasm of the infected cell is disorganized as compared to that of WI-38 control cells (inset). X 15,000. Inset, X 15,000.

Fig. 8. The nuclear inclusion body of a skin-like appearance is associated with viral precursors. Some are empty shells consisting of 1 membrane; others are double shelled. X 36,000.

Fig. 9. The nuclear inclusion body (IB) is composed of a fibrillar viral matrix. Shells with 1 or 2 membranes (arrows) are present, and incomplete particles containing electron-dense centers are frequent. The latter are surrounded by a single membrane. X 36,000.

Fig. 10. Incomplete virus particles are present in the perinuclear space (arrow), budding through the cytoplasmic matrix into vacuoles (inset), and infrequently budding at the cell membrane into intercellular spaces. X 62,000. Inset, X 70,000.

Fig. 11. WI-38 cells 8 dpi. The nucleus is filled with numerous incomplete virus particles. The cytoplasm is disorganized, and virus particles at different stages of development (arrows) are accumulating at the cytorectum. Few lysosomes (L) are present. X 12,000.

Fig. 12. WI-38 cell 8 dpi. The cytoplasm adjacent to the Golgi area (G4) is filled with virus particles in close association with lysosomal bodies. In some instances, virus particles and lysosomes are found in the same membrane-enclosed structure (inset). X 25,000. Inset, X 30,000.
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