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Human Cytomegalovirus: Development and Progression of Nuclear Inclusions by Primary Clinical Isolates and Laboratory-Adapted Strains (42274)

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Abstract. The morphogenesis of cytomegalovirus (CMV) nuclear inclusions (NIs) was investigated using unadapted clinical isolates and adapted laboratory strains. Both adapted and unadapted strains of CMVs induced NIs whose morphologic appearance was similar in human fibroblastic cells. Early NIs appeared as ring-like structures composed of dense granular and fibrillar material, while late NIs appeared to consist of multiple electron-lucent areas containing coarse granules and bounded by electron-dense fibrillar material (cellulae). Capsids and nucleocapsids were associated primarily with the electron-dense fibrillar material; however, developing nucleocapsids were most often observed at the interface of the electron-dense and -lucent areas. Although there was some variation in the rate of development and maturation of the NIs with the intensity of infection, all CMVs examined produced late NIs with similar organizational patterns consisting of cellulae. Substitution of human fibroblastic cells derived from various tissues as cellular substrate did not appreciably affect the results. Thus, the unique organization of the CMV late NI, consisting of multiple cellulae, appears to be an intrinsic feature of CMV replication since it seems to be independent of the extent of laboratory adaptation, the virus strain, the intensity of infection, or the cell type.

Unique aspects of the morphogenesis of nuclear inclusions induced by cytomegalovirus (CMV) as detected by light and electron microscopic techniques have been recently emphasized (1, 2). It was reported that early nuclear inclusions (NIs) appeared as bead-like structures by light microscopic studies and as ring-like structures composed of dense granular and fibrillar material by ultrastructural analysis. In these studies, late NIs were found to develop separately from the early NIs and to consist of multiple subunits that we have termed cellulae. Although interesting, these observations may have limited applicability since they are based on a single laboratory-adapted strain, AD169, at a constant, high multiplicity of infection (MOI).

It could be questioned, in light of the extent of biological and biophysical variation detected among human CMV isolates for more than three decades, whether such observations made with a single CMV isolate after scores of subcultures are representative of CMVs in general. These variations include differences in plaque morphology (3), the development and progression of cytopathic effects (1), antigenic heterogeneity (4), differential neutralization by antibody (5), the kinetics of DNA–DNA renaturization (6), and analysis of restriction endonuclease fragments (7). The present study was undertaken to determine if the unique features described for CMV-induced NIs were independent of the CMV strain, the intensity of infection, or the cell type, and if they are representative of both primary clinical isolates and laboratory-adapted CMV strains.

Materials and Methods. *Cell cultures.* Human embryo lung (LU), thyroid (THY), and skin muscle (SM) cells in the 10th to 25th passage were used. Morphologic (1, 2) and biologic features of the interaction of these fibroblasts with CMV have been previously described [for a review see Ref. (8)]. The methods of initiating and maintaining these cell cultures were described in detail previously (3). No contamination by mycoplasma was detected in cell cultures when tested by the method of Clyde and Kim (9).

Virus stocks. The strains of CMV studied in this report were AD169 (10), C87 (11), Davis (12), 82-1, 84-2, and 85-1. AD169, C87, and Davis are all common laboratory strains

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for which we and others have described numerous features. CMV strain 82-1 was recovered from the liver and lung tissue of a patient who succumbed to disseminated CMV infection following renal allograft. Frozen specimens of lung or liver homogenate were used as inocula. CMV strains 84-2 and 85-1 were isolated from the urine of congenitally infected children. Frozen specimens of urine were used as inocula. Isolates 82-1, 84-2, and 85-1 were identified as CMVs by induction of characteristic cytopathology, and by indirect immunofluorescence assays of infected cells using CMV-specific antibody. Gentamicin (20 μg/ml) was added to cell cultures inoculated with the clinical isolates.

Electron microscopy. Cultures of fibroblast cells were prepared on coverslips as described previously (1). Mock-infected (uninfected cell lysate or media) cells were used as controls. At various time intervals, the coverslip cultures were removed from the maintenance media and washed once in phosphate-buffered saline (PBS), pH 7.2. The cells were fixed *in situ* with glutaraldehyde (2). Cell monolayers, gently removed from each coverslip with a clean blade, were pelleted in microfuge tubes, postfixed with 1% osmium tetroxide (2 hr), and en bloc stained with 1% uranyl magnesium acetate (overnight). The pellet was rinsed in distilled water, dehydrated through a graded ethanol series, and embedded with epoxy resin. Thin sections, cut with a diamond knife on an LKB ultramicrotome, were stained with lead citrate, and examined and photographed with a Phillips 201 Electron microscope.

Light microscopy. At selected time intervals, coverslip cultures of infected cells were washed twice in PBS and fixed in Bouin's solution (1). The cells were then stained with Harris' hematoxylin (20 min) and alcoholic eosin (10 min). CMV-infected and control cells were examined and photographed with a Zeiss photomicroscope III.

Indirect immunofluorescence. Following acetone fixation at 0°C, cells on 18-mm-diameter coverslips were exposed for 30 min (37°C) to a CMV-reactive or CMV-nonreactive human convalescent sera. After three rinses in PBS, the cells were exposed for 30 min (37°C) to light and heavy chain-specific goat anti-human IgG that had been conjugated to fluorescein isothiocyanate. Cells were air-dried and mounted on microscope slides with PBS/Glycerol (1/1) and examined with a Zeiss photomicroscope III equipped for fluorescence detection.

Results. Our previous light microscope studies (1) suggested that the development and progression of NIs were similar among five laboratory-adapted strains of CMV. In the present ultrastructural study, LU cells infected with CMV strains C87, Davis, and AD169 exhibited comparable NIs at 96 to 144 hr postinfection (hr PI), as shown in Fig. 1. These late NIs contained a network of dense, fibrillar, branching material often enclosing electron-lucent areas. The interior of these cellulae contained coarse granular material. Nucleocapsids were present primarily in the electron-dense fibrillar network. At the interface of the electron-dense and electron-lucent components of the late NIs, dark structures with tapering tails were frequently associated with virus particles (Fig. 2). Often large dense bodies were found at the periphery of the NIs. It appears, therefore, that the ultrastructural features of the CMV NI are common to at least three laboratory strains.

The dependence of the observed ultrastructural characteristics on the intensity of infection (gene dose) was examined in cells infected with a wide range of calculated MOIs. LU cells infected with 0.5, 5, or 50 plaque-forming units (PFU)/cell of CMV (strain AD169) exhibited NIs that were ultimately indistinguishable by light and electron microscopy from those described previously, although the development and progression of NIs were somewhat faster with greater multiplicities. The mature NIs, as shown in Fig. 3, were first observed in cells infected at 0.5 PFU/cell at approximately 125 hr PI. At 5 PFU/cell similar NIs were first observed at 96 hr PI, and at 50 PFU/cell at 72 hr PI. Similar results were obtained with the CMV strain Davis at multiplicities of 0.5, 5, and 50 PFU/cell. The intensity of infection appeared to have no substantial effect on the overall organization and structure of the resultant NIs.

Human cells derived from various tissues have been used to study CMV replication. These cells may represent an important variable that could influence the structure and development of CMV NIs. The development and progression of the late NIs, however, were

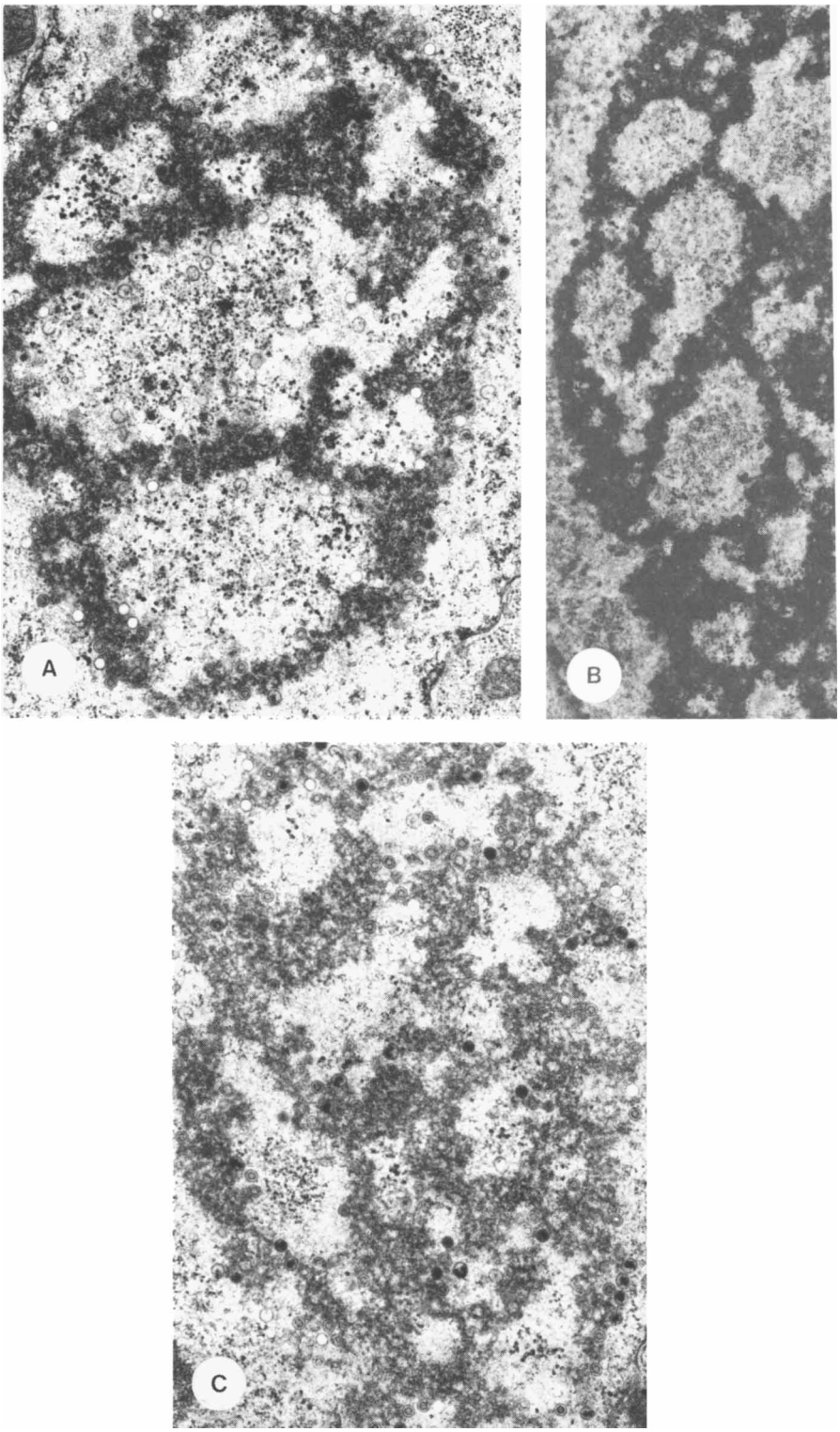

FIG. 1. The late CMV nuclear inclusion induced by 3 laboratory strains in human embryo thyroid cells. (A) C87, 5 PFU/cell, 125 hr PI ×16,430; (B) Davis, 3 PFU/cell, 144 hr PI ×11,210; (C) AD169, 50 PFU/cell, 144 hr PI, ×16,430.

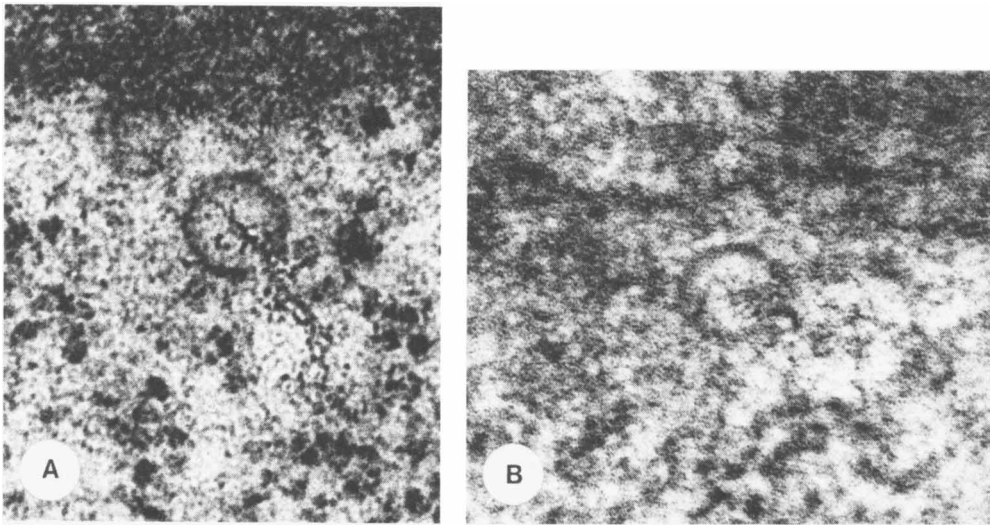

FIG. 2. Electron micrograph of CMV-infected cells. The fusiform structures are associated with incomplete virus particles at the interface of the electron-dense and electron-lucent areas within the late NI. (A) AD169, 96 hr PI, THY cells, ×112,500; (B) isolate 82-1, 216 hr PI, LU cells, ×120,000.

similar when observed in LU, THY, or SM cells infected with the various strains of CMV at MOIs of about 5 PFU/cell when studied by light microscopic technique (1). In this study no substantial ultrastructural differences were observed in the NIs induced by strain AD169 in the several cell types (data not shown). Both the size and organization of the NIs and the relative populations of virus particles were similar. Therefore, the source of the human fibroblastic substrate used to propagate CMV does not appear to have a discernible effect on the structure of the resultant CMV-induced NIs.

To determine the effect of laboratory adaptation on the development and progression of the CMV NIs, LU cells were inoculated with unadapted primary clinical isolates of CMV contained in tissue homogenate (82-1), or urine (84-2 and 85-1). By light and electron microscopic studies, the general appearance of the late NIs induced by these unadapted CMV isolates was similar to that observed with laboratory-adapted strains of CMV (Figs. 4 and 5). Hematoxylin and eosin-stained cells infected with these CMV isolates appeared to have NIs consisting of cellulae comparable to those found in cells infected with laboratory-adapted strains (Fig. 4). The cellulae consisted of a branched network of electron-dense material enclosing electron-lucent areas (Fig. 5). The distribution of virus particles was similar to that observed with the adapted strains. Nucleocapsids and capsids were observed primarily in the electron-dense network, while virus particles composed of apparently incomplete capsids and dark structures with tapering tails were most frequently observed at the interface of the electron-dense and -lucent areas. Mature NIs first appeared at approximately 120 hr PI. Although the NIs induced by the primary CMV isolates appeared to be smaller, less dense, and less organized than those induced by laboratory-adapted strains, the general features of development, organization, and progression were similar to those observed with adapted strains.

Discussion. The present study suggests that the structure and organization of the CMV NIs reported previously for strain AD169 (2) are similar to that induced by other laboratory strains of CMV and by unadapted clinical CMV isolates. The structural organization and developmental pattern of the CMV NIs do not appear to be dependent on laboratory adaptation, since three wild strains induced the formation of NIs that were similar to those induced by adapted strains. The NIs described by Kanich and Craighead (13) induced by a wild strain of CMV are similar to those observed in this study. Thus, the fundamental structure of the CMV NI does not appear to be dependent on the CMV strain or the level of cultural adaptation of the CMV isolate.

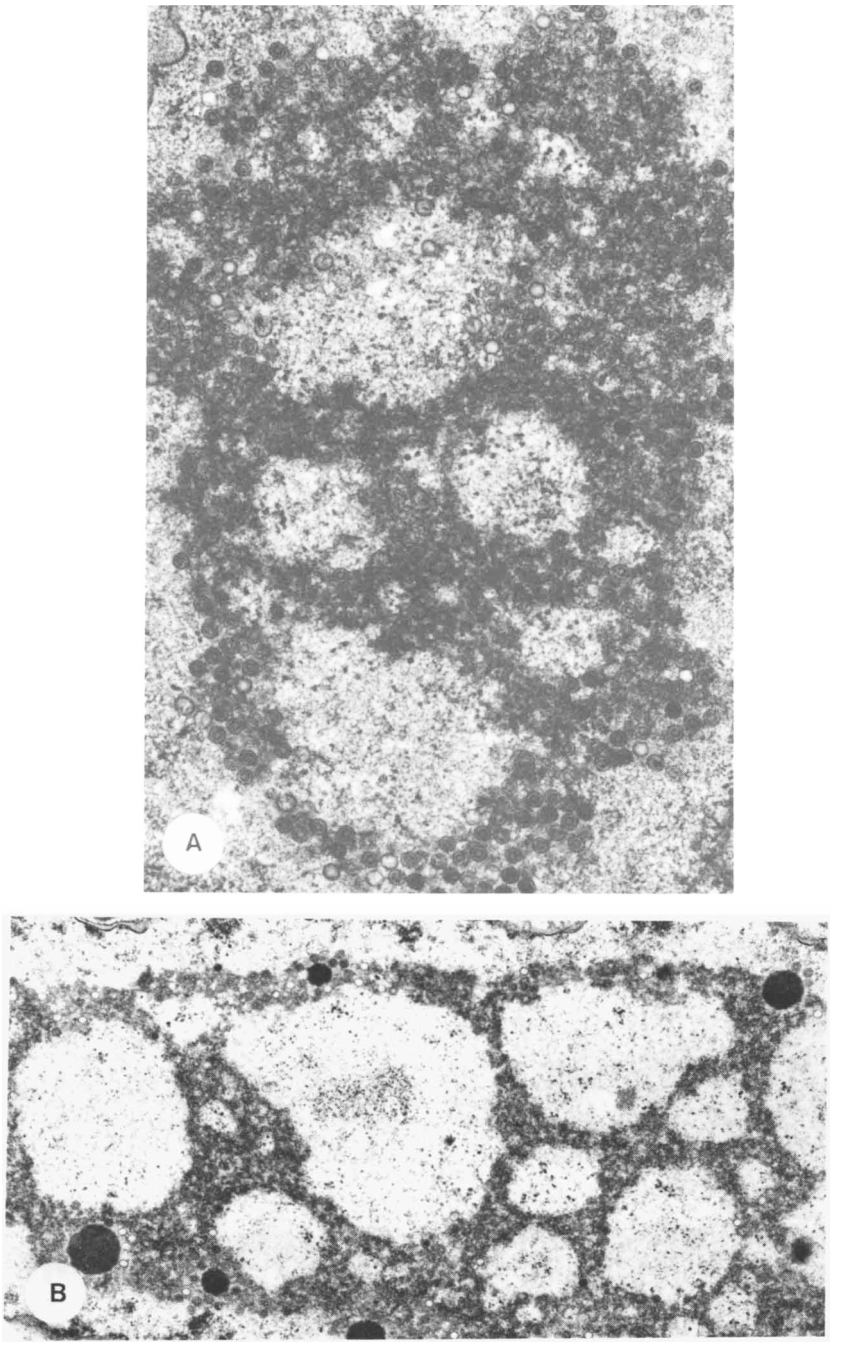

FIG. 3. Development of the CMV (strain AD169)-induced late nuclear inclusion as a function of the multiplicity of infection in THY cells. (A) 0.5 PFU/cell, 125 hr PI, ×17,290; (B) 5.0 PFU/cell; 144 hr PI, ×11,800.

The source of the fibroblastic cells used for these studies (lung, skin muscle, thyroid) did not appreciably alter the development and progression of the NIs. This result was consistent with earlier studies demonstrating that the susceptibility and time course of CMV replication were similar in these cell types (1, 3, 14).

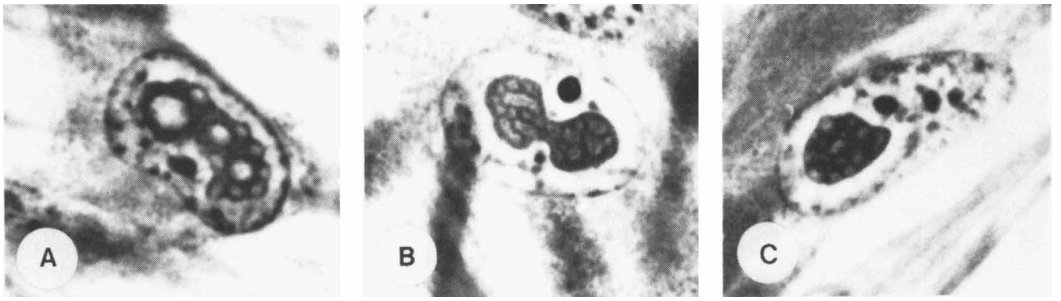

FIG. 4. The NIs induced by unadapted clinical isolates of CMV. (A) Strain 85-1, SM cells, (B) Strain 84-2, LU cells, (C) 82-1, LU cells, H and E. A, B, C, ×780.

The rate of development and progression of the NIs was found to be dependent on the MOI. Dependence of the rate of development and progression of the NIs on the MOI would be expected as a consequence of the virus gene dose. It has been suggested previously that some of the variation in the degree of organization of the CMV NIs at a given time may result from the use of various MOIs (1). The present results reinforce that concept, although they do not explain the observations of Smith and deHarven, who obtained different results with similar MOIs (15).

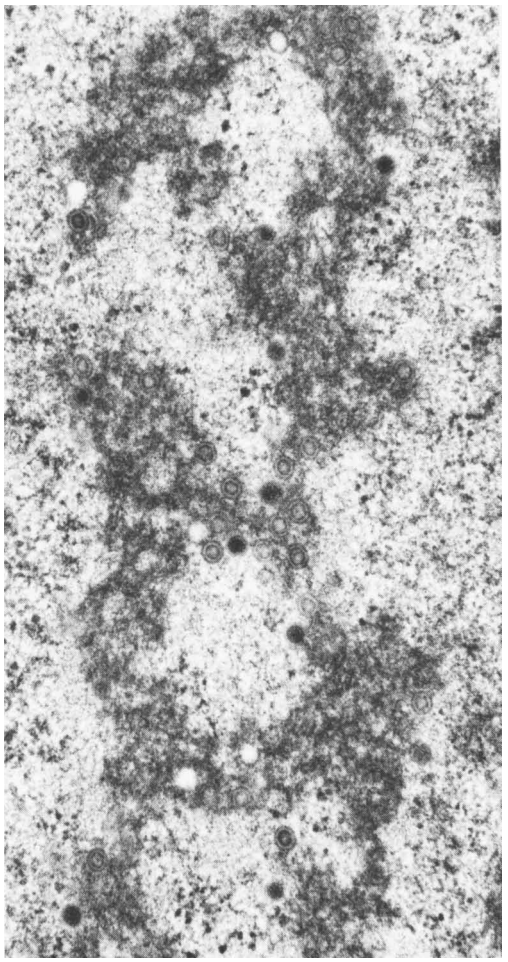

FIG. 5. The late CMV nuclear inclusion induced by an unadapted isolate (Strain 82-1) at 96 hr PI in LU cells. ×29,230.

Although the structure and development of the NIs described above and previously (1) are common to all CMV strains studied to date, the organization of the NIs into cellulae appears to be unique to CMVs. Similar NIs have not been observed with herpes simplex virus or varicella zoster virus infections. It is possible that the unique structure of the CMV NIs results from the overall slow rate of CMV replication. Alternatively, the organization of the CMV NIs into cellulae may result from patterns of molecular regulation of virus replication that are distinct to CMVs and also result in relatively slow replication. Additional work will be required to distinguish between these possibilities.

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