Cytomegalovirus: An Ultrastructural Study of the Morphogenesis of Nuclear Inclusions in Human Cell Culture

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(Accepted 14 April 1981)

SUMMARY

We investigated the ultrastructural development and maturation of cytomegalovirus (CMV) nuclear inclusions (NIs) in human embryo thyroid cells at 1 to 144 h post-infection. At 5 h, most cells had rounded from an initial fibroblastic appearance and contained early NIs. At 24 h, early NIs were larger and better defined. At 48 h, although early NIs were still present, most cells had larger and presumably more mature NIs. These latter NIs consisted of several subunits, each made up of a fibrillar network enclosing an electron-lucent area which contained coarse and delicate granules. Also, at 48 h, virus particles were first seen in the nucleoplasm. At 72 h, in cells with more developed NIs, virus particles were closely associated with the fibrillar network. Between 96 and 144 h, the NIs reached maximum size and were made up of numerous subunits. The results indicate that two types of NIs coexist during CMV infection. The appearance of the early and late NIs coincides with the reported peaks of CMV DNA synthesis and thus may explain the biphasic pattern of DNA synthesis in CMV infection. Morphogenetic features of the NIs conform with the hypothesis that synthesis of CMV DNA may occur in the centre of each NI subunit and that the fibrillar network represents condensing capsid proteins.

INTRODUCTION

Human disease associated with cytomegalovirus (CMV) infection was first recognized by the unique cytopathology induced in host tissues (Jesionek & Kiolemenglou, 1904). The principal cytopathic effects of CMV infection are the formation of nuclear and cytoplasmic inclusions (Smith, 1956; Rowe et al., 1956; Weller et al., 1957).

In a previous report (Albrecht et al., 1980a) we presented data indicating that the nuclear inclusions (NIs) in CMV-infected cells consist of a variable number of associated subunits. When fully developed, each subunit appears as an oblong or round structure with a central darkened area; with further morphogenesis, there is collapse and eventual fragmentation of the NI. The present study had two objectives: to examine the ultrastructural morphogenesis of the NIs and to attempt to reconcile reported patterns of CMV DNA synthesis with morphogenetic features of the NIs.

METHODS

Cell cultures. Human embryo thyroid cells in the 5th to 23rd passage were used. The methods of initiating and maintaining the cell cultures were as described previously (Albrecht & Weller, 1980). The cell cultures exhibited no contamination by mycoplasma when tested by the method of Clyde & Kim (1967).

Virus stocks and plaque assay. Strain AD169 (Rowe et al., 1956) of human CMV was used. Stocks of this strain were prepared as described previously (Albrecht & Weller, 1980).
The infectivity of virus stocks was measured by a modification (Albrecht & Rapp, 1973) of the agarose overlay plaque technique of Wentworth & French (1970). The stock of the AD169 strain of CMV yielded a multiplicity of infection of 47.5. The virus recovered its input infectivity between 49 and 52 h. Maximum and consistent virus yields occurred from 96 to 144 h.

**Morphologic studies.** Cultures of thyroid cells were prepared on coverslips and infected as described previously (Albrecht et al., 1980a). Uninfected cells were used as controls. At various time intervals (1, 5, 24, 48, 72, 96, 120 and 144 h) cell cultures were removed from the maintenance fluids and washed once in phosphate-buffered saline pH 7.2. The cells were then fixed in situ with 2% glutaraldehyde. Cell monolayers, gently removed from each coverslip, were pelleted by centrifugation and processed routinely for electron microscopy.

**RESULTS**

At 1 h, cell morphology was unchanged, compared to controls; numerous virus particles were present in the cytoplasm. At each of the remaining time intervals, a range of cytopathic effects was observed. The description that follows represents the cytopathic changes observed most commonly.

At 5 h, most cells had rounded (Fig. 1). In most nuclei, a ring-like structure (early NI) (Fig. 1, arrowheads), composed of granular and fibrillar material, distinct from the nucleolus, was first seen. The nucleoplasm contained grainy areas. Virus particles were often contiguous with the outer leaflet of the nuclear envelope (Fig. 1, inset). The nuclei demonstrated an irregular profile, and the chromatin was clumped and marginated.

At 24 h, most cells had returned to their fibroblastic appearance. The early NI was larger, more fibrillar, and better defined (Fig. 2, arrowheads). Virus particles were still seen in the cytoplasm but not in the nucleus. Clumping of the nuclear chromatin was less prominent. The pars fibrosa of the nucleolus was enlarged in most cells.

At 48 h, all cells had a fibroblastic appearance. The NIs were in various stages of development, denoted by their size and organization and by the presence, number, and location of virus particles (Fig. 3). Although early NIs were still present (Fig. 3, arrowheads), most cells now contained larger NIs. The larger NIs were made up of a network of dense, fibrillar, branching material, often enclosing electron-lucent areas (Fig. 3). For each structure constituted of a fibrillar network enclosing an electron-lucent area, the term ‘cellula’ is adopted. Nucleocapsids (Fig. 3, inset) were observed in most nuclei. When present in small numbers, such virus particles were seen at the periphery of the nucleus. When in large numbers, virus particles were located preferentially at the vicinity of NIs or within the cellulae. Margination of chromatin was not a prominent feature. In most nuclei multiple areas of closely packed, delicate, grainy material were present (not illustrated at this time interval).

At 72 h, both the nucleus and the NIs were very large. The number of virus particles within the fibrillar component of the cellulae was increased (Fig. 4). Occasionally, fusiform or elongated dark structures with tapering tails were seen at various stages of incorporation within virus particles (Fig. 5). The interior of the cellulae showed large amounts of coarse, granular material. Virus particles and virions (not illustrated) were now seen in the cytoplasm. Other nuclear findings were as at 48 h.

At 96 and 120 h, findings were comparable. The NIs were composed of large numbers of cellulae which often contained a central darkened area (Fig. 6, arrows). The number of virus particles was increased. Areas of grainy material (Fig. 6, asterisks) were larger and better defined. Large, dense, spherical bodies were often seen at the periphery of the nuclei (Fig. 6). The nuclear chromatin was marginated in large clumps. Other nuclear findings were as at 48 h.
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Fig. 1. Cell rounding at 5 h. An early NI (arrowheads) is evident. Inset: virus particle contiguous with the outer leaflet of the nuclear envelope; nc, nucleolus. Fig. 1, bar marker represents 1 μm; inset, bar marker represents 0.1 μm.

Fig. 2. Cell with fibroblastoid appearance at 24 h. The early NI (arrowheads) is large and well defined; nc, nucleolus. Bar marker represents 1 μm.

Fig. 3. NIs at 48 h. An early NI (arrowheads), constituted predominantly of a fibrillar network, is demonstrated. A larger and presumably more mature NI, composed of a branching fibrillar network (FN) that demarcates one or more electron-lucent areas, is evident. The inset is a detail of the virus particles; nc, nucleolus. Fig. 3, bar marker represents 1 μm; inset, bar marker represents 0.1 μm.

At 144 h, the NIs occupied most of the nucleus and the fibrillar network of each cellula was populated by a massive number of virus particles (Fig. 7). Although the central darkened area still persisted in some cellulae, more often large globular deposits were embedded within
Fig. 4. NI at 72 h. The thick fibrillar network (FN) shows numerous associated virus particles. The center of each of the cellula contains abundant granules. Bar marker represents 1 μm.

Fig. 5. Detail of Fig. 4 to show fusiform, or elongated dark structures (arrows) incorporated within virus particles at the interface of the fibrillar network (FN) and the electron-lucent area of the cellulae. Bar marker represents 0.1 μm.

Fig. 6. NI at 96 h. Note darkened areas (arrows) in the center of the cellulae. Areas of grainy material (asterisks) near the NI are well demarcated. Large spherical bodies (SB) are evident. Bar marker represents 1 μm.
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Fig. 7. NI at 144 h. The fibrillar network (FN) is packed with capsids and nucleocapsids. Bar marker represents 1 µm.

Fig. 8. NI at 144 h. Electron dense, globular (G) material is seen embedded in the fibrillar network (FN). Bar marker represents 1 µm.

Fig. 9. Condensation of NI at 144 h. Bar marker represents 1 µm.

Fig. 10. Virus particles surround a condensed NI at 144 h; nc, nucleolus. Bar marker represents 1 µm.

the fibrillar network (Fig. 8). The coarse, granular material in the centre of the cellulae was still prominent (Fig. 7 and 8). In a large number of cells, the NIs were condensed; the cellulae were partially collapsed and confluent, which resulted in aggregates of various sizes (Fig. 9). Although condensation of the NIs occurred while virus particles remained associated with the fibrillar network (Fig. 9), in a small number of cells, virus particles surrounded the condensed NIs (Fig. 10).
DISCUSSION

In a previous report (Albrecht et al., 1980a), light microscopic observations suggested that CMV NIs were present by 24 h. Our electron microscopic studies demonstrate that structures which represent early NIs were, in fact, detectable in most cells by 5 h. This occurred much earlier than previously reported (McAllister et al., 1963; Rapp et al., 1963; Goodheart et al., 1964; McGavran & Smith, 1965; Ruebner et al., 1965; Heine et al., 1971; Kanich & Craighead, 1972; Iwasaki et al., 1973; Smith & de Harven, 1973). The presence of early NIs at this time is consistent with the biochemical demonstration that early CMV proteins are synthesized before 5 h (Furukawa et al., 1973; Geder, 1976; Reynolds, 1978; Stinski, 1978).

Since CMV DNA synthesis has not been detected biochemically before 10 h (Stinski, 1978), two possibilities arise: (i) that virus DNA synthesis may begin after formation of the early NI, or (ii) that virus DNA synthesis may occur in early NIs at levels not detectable by the methods used.

NIs progress from the structure containing fine and coarse granular material enclosed by an electron-dense fibrillar network (cellula) to NIs composed of multiple cellulae (48 to 96 h). The fibrillar network of mature NIs (Fig. 4 to 7) has frequently been considered to be a skein of virus DNA or chromatin-like material (Luse & Smith, 1958; Smith, 1959; McGavran & Smith, 1965; Martin & Kurtz, 1966; Smith & de Harven, 1973). Current and previous (Albrecht et al., 1980a) morphological studies from our laboratory suggest an alternative explanation. First, the morphology of the CMV NI is the result of associated cellulae. Second, the central, electron-lucent area of the cellulae could represent an area of active virus DNA synthesis. Third, the fibrillar network delimiting individual and anastomosing cellulae is more likely to represent condensing virus structural proteins. Accordingly, one would expect to find developing capsids in this area and the filling of capsids with nucleoprotein at the interface of the fibrillar and electron-lucent portions of the cellulae. Such findings were demonstrated (Fig. 4 and 5) in our studies.

The progression of the NIs from a single cellula to multiple cellulae suggests that synthesis of CMV DNA is confined to discrete areas of replication. The molecular basis by which this compartmentalization is accomplished is not presently understood. Nevertheless, condensation of virus structural proteins around areas of virus DNA synthesis could result in the observed cellula structures. The biphasic nature of CMV DNA synthesis (Albrecht, 1973; St. Jeor & Hutt, 1977; Stinski, 1978) could be explained by the initial synthesis of DNA within the early NI to form the replicative centres of the cellulae, followed by a second cycle of DNA synthesis directed by the earlier DNA copies. The timing of the CMV DNA synthesis cycle as reported by either St. Jeor & Hutt (1977) or Stinski (1978) conforms with both our results and this latter interpretation. If this hypothesis of the sequence of CMV DNA synthesis and the composition of the cellulae is correct, then CMV replication is distinct from, and perhaps more complex than, that of either herpes simplex virus or varicella-zoster virus where we have not observed similar structures (T. Albrecht, T. Cavallo, N. L. Cole & K. Graves, unpublished observation).

The question then arises as to why viruses that are closely related, and presumed to have similar modes of replication, should induce such diverse cytopathic effects. One possibility is that synthesis of CMV DNA and that of structural protein are relatively asynchronous. Such a view would be supported by the relationship of nucleocapsids to the fibrillar network of the cellulae, which suggests that CMV genomes are in excess early in the infection and in short supply late in the replication cycle, relative to virus structural proteins. For example, at 48 h (Fig. 3), nucleocapsids are scattered about the nucleoplasm. By 72 h (Fig. 4), the majority of the nucleocapsids are found in the fibrillar network of the cellulae. Late, at 96 to 144 h, when CMV DNA synthesis is declining (Albrecht et al., 1980b), nucleocapsids are also seen near the centre of the cellulae, at a time when numerous large dense bodies of what could represent
excess structural proteins are present in the nucleus. Cytoplasmic dense bodies with similar characteristics have been reported (Craighead et al., 1972; Kanich & Craighead, 1972; Sarov & Abady, 1975, 1977; Stinski, 1976; Smith & de Harven, 1978; Albrecht et al., 1980a), and have been demonstrated to consist of virus structural proteins (Craighead et al., 1972; Sarov & Abady, 1975; Stinski, 1976). If, in fact, as our observations suggest, structural proteins relative to CMV genomes are scarce early in the infection and in excess later, this seemingly dis coordinated macromolecular synthesis would provide increased opportunity for restriction of virus replication and for formation of persistent infections (Weller, 1971; Rapp et al., 1975; Li & Albrecht, 1979; Mocarski & Stinski, 1979; Albrecht et al., 1980b), an evolutionary advantage to CMV.

Cytomegalovirus strain AD169 was provided by Dr T. H. Weller. This work was supported in part by the W. L. Moody Foundation, by the James W. McLaughlin Fellowship Fund, and by Grant AM-27684 from the National Institute of Arthritis, Metabolism, and Digestive Diseases. The authors gratefully acknowledge the editorial recommendations of Mrs Marilyn Thompson.

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(Received 20 September 1980)