An Ultrastructural Investigation of Cytomegalovirus Replication in Murine Hepatocytes

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SUMMARY

The morphological characteristics of murine cytomegalovirus replication in murine hepatocytes were investigated by electron microscopy and structural evidence of an unusual mode of virus maturation was detected. Nucleocapsids form in the nucleus; those with electron-dense cores bud into the perinuclear cisternae and acquire an outer envelope from the inner membrane of the nuclear envelope. Viral envelopes fuse with the outer membrane of the nuclear envelope releasing nucleocapsids in the paranuclear zone where they aggregate and form cytoplasmic inclusions. These inclusions are invariably surrounded by lamellae and vesicles of the Golgi complex into which nucleocapsids again bud. Virions, now covered with a new membrane envelope, are transported within secondary lysosomes and released by emiocytosis into the extracellular space.

INTRODUCTION

The cytomegaloviruses are included in the herpesvirus group and can infect a variety of animal species. In man they may be responsible for congenital and neonatal infections (Stern et al., 1969; Reynolds et al., 1973, 1974), a variety of clinical syndromes in children and adults (Diosi & Rosin, 1965; Klemola & Kääriäinen, 1965; Carter, 1968; Henson, 1969; Klemola et al., 1969; Craighead, 1971; Weller, 1971a, b), acquired immune deficiency (Duwall et al., 1966; Rinaldo et al., 1980; Drew et al., 1981; Mildvan et al., 1982), and may complicate organ transplantation (Craighead et al., 1967; Spencer, 1974) and open heart surgery (Kääriäinen et al., 1966; Lang et al., 1968; Caul et al., 1971).

Cytomegalovirus infection in other animals produces pathological lesions resembling those of the human disease and provides useful models for understanding the infectious process. Depending on the dose of virus, experimental infection of mice with murine cytomegalovirus (MCMV) can result in a fatal disease with widespread and extensive visceral lesions or a chronic infection of various organs with virus persisting especially in the salivary glands (McCordock & Smith, 1936; Smith, 1954; Brodsky & Rowe, 1958; Mannini & Medearis, 1961; Medearis, 1964; Henson et al., 1966; Henson & Neapolitan, 1970; Henson & Strano, 1972; Lussier et al., 1974; Larson, 1976; Olding et al., 1976; Smith & Wehner, 1980; Grundy et al., 1981; Papadimitriou & Shellam, 1981). The liver, spleen, lymphoid tissues, heart, kidney, bone marrow as well as the salivary glands are usually invaded by MCMV in the course of the disease.

In this report we have examined the ultrastructural features of MCMV replication in murine hepatocytes and documented an unusual maturation process. The structural events in the development of the virions beginning with the formation of nucleocapsids in the nucleus, their maturation in the cytoplasm and their egress into the extracellular space have been elucidated, while the mode of formation of the cytoplasmic inclusions (Rowe et al., 1956; Weller et al., 1957; Smith, 1959; McGavran & Smith, 1965; Ruebner et al., 1966) has been partly characterized. Lastly the structural lesions in lethally infected normal mice and in those heterozygous (bg+/) and homozygous (bg/bg) for the beige mutation have been compared. The latter were chosen because this mutation is a homologue of the Chediak–Higashi syndrome in man (Lutzner et al., 1967; Bennett et al., 1969) in which lysosomal anomalies have been reported (Prieur et al., 1972; Kimball et al., 1975; Vassalli et al., 1978).
METHODS

Animals. BALB/c, C57BL/6J bg/ + (bg/+ ) and C57BL/6J bg/bg (bg/bg) mice were obtained from highly inbred stock at the Animal Resources Centre, Murdoch, Western Australia. Female mice aged 10 to 12 weeks were infected with a lethal dose of MCMV. Five mice of each strain were sacrificed on the second, third and fourth day after infection and samples of liver taken for electron microscopy. Similar samples from the livers of three normal mice from each strain were used as controls.

Virus. Virus stocks and appropriate inocula were prepared as previously described (Shellam et al., 1981; Papadimitriou et al., 1982). All inoculations were given intraperitoneally in 0·1 m-phosphate-buffered saline. A lethal dose of 3 x 10^5 p.f.u. was given intraperitoneally to each bg/+ mouse while BALB/c and bg/bg mice received 10^4 p.f.u. each.

Electron microscopy. Samples of liver were fixed in cold 2·5% glutaraldehyde in 0·1 N-phosphate buffer (pH 7·4). After 16 h the tissues were washed in phosphate buffer and post-fixed in 1% osmium tetroxide in 0·1 m-phosphate buffer, dehydrated in graded solutions of ethanol and embedded in Araldite. Sections were cut on an LKB ultramicrotome, stained with lead hydroxide and examined in a Philips 201 electron microscope.

RESULTS

No major ultrastructural differences were detected between the cells supporting MCMV replication in the livers of the BALB/c strain and those of bg/bg and bg/+ mice, although electron-dense cytoplasmic inclusions were somewhat more frequent in hepatocytes of bg/bg mice. In addition, the range of ultrastructural lesions on any two successive days overlapped somewhat, but, overall, hepatocytes with structural evidence of virus replication were more frequently encountered in livers 3 and 4 days after infection while necrotic cells were easily detected on the fourth day. Lastly, no convincing evidence of virus replication in Kupffer cells or other sinusoidal cells was found and MCMV production was predominant in hepatocytes.

Two days after infection, hepatocytes in which there was evidence of virus replication displayed enlarged nuclei with mildly irregular contours. The amount of euchromatin had increased, nucleoli were prominent and often near the nuclear envelope, while the heterochromatin was confined to a thin rim at the nuclear periphery (Fig. 1). Within the euchromatin, patches of amorphous electron-dense matrix and aggregates of tangle electron-dense fibrillogranular material were present (Fig. 1), the fibrils in the latter measuring 9 to 10 nm in diameter. In addition, many viral nucleocapsids were scattered throughout the euchromatin (Fig. 1). The majority of these displayed spherular nucleoids enclosed by a peripheral capsid but a few appeared structurally malformed, displaying incomplete capsids. The complete nucleocapsid measured 95 to 100 nm in diameter while the enclosed nucleoid had a diameter of 50 to 55 nm. Most nucleocapsids possessed poorly osmiophilic spherular nucleoids but a few exhibited extremely electron-dense nucleoid cores. The latter were situated at the periphery of the fibrillogranular aggregates or within or at the periphery of the amorphous electron-dense matrix (Fig. 1).

Membrane-coated particles, measuring 110 to 130 nm in diameter, were present within the perinuclear cisternae of the nuclear envelope. Many contained obvious nucleocapsids in which the nucleoid was often electron-dense, others possessed only a spherule of capsid-like material, while a few did not appear to enclose any recognizable viral structures (Fig. 1). In addition, an

Fig. 1. Hepatocyte 2 days after infection with MCMV. Nuclei display irregular contours and prominent nucleoli. Note the presence of electron-dense matrix (ED) and fibrillogranular material (FG) in the nuclear sap. Viral nucleocapsids are scattered in the nucleus while amorphous electron-dense material is seen in the perinuclear cytoplasm (arrow). Bar marker represents 1 μm.

Fig. 2. Nucleus of a hepatocyte 2 days after infection with MCMV. Some of the particles in the perinuclear cisternae are seen fusing with the outer membrane of the nuclear envelope (arrows). The latter surrounds a ribosome-rich cytoplasmic invagination in which nucleocapsids are surrounded by an osmiophilic cloud. The curved arrow shows a nucleopore. Bar marker represents 200 nm.

Fig. 3. Hepatocyte 3 days after infection with MCMV. Some of the particles in the perinuclear cisternae (arrows) consist only of perinuclear fibrous lamina, a coreless nucleocapsid and the inner membrane of the nuclear envelope. In addition, two nucleocapsids (curved arrows) are seen budding into the perinuclear cisternae. Inset shows a tail-like projection on an intracisternal particle. Bar markers represent 200 nm.
amorphous electron-dense matrix was seen in the perinuclear cytoplasm and in the cytoplasmic invaginations which indented the nuclear periphery (Fig. 1, 2). These invaginations contained not only the electron-dense matrix but also many ribosomes and occasionally finely fibrillar material (Fig. 2). Nucleocapsids were often embedded within the amorphous electron-dense matrix in the perinuclear cytoplasm and almost all displayed electron-dense nucleoids (Fig. 2).

In the cytoplasm of these infected hepatocytes the cisternae of the rough endoplasmic reticulum (RER) were prominent while mitochondria were somewhat enlarged but otherwise intact. Lipid globules and glycogen rosettes were less prominent than in uninoculated controls.

By the third day of infection the nuclear contours of infected cells were more irregular than those encountered on the second day. Deep cytoplasmic invaginations were frequently detected within the nucleus while flap-like extensions of the nuclear envelope often projected into the perinuclear cytoplasm which contained many ribosomes and an amorphous electron-dense matrix. Within the nucleus the number of nucleocapsids had increased but the proportion of those with electron-dense cores had not altered, while patches of amorphous electron-dense matrix were frequently found in the nucleoplasm. Membrane-coated particles were frequent within the perinuclear cisternae of the nuclear envelope and again those with electron-dense nucleocapsids predominated, although membrane-enclosed coreless capsids were also seen (Fig. 3, 4). Rarely, the membrane-coated particles displayed a small tail-like protrusion attached to the common circular profile (Fig. 3). Occasionally, these intranuclear cisternal particles were seen budding from the nucleoplasm, carrying with them the inner membrane of the nuclear envelope (Fig. 3, 4). Although this process was most frequent in areas where cytoplasmic invagination of the nucleus had occurred it was also observed in the more convex regions of the nuclear periphery.

At the periphery of the cytoplasmic inclusions the membrane of some intranuclear cisternal particles was seen fusing with the outer membrane of the nuclear envelope, releasing the enclosed capsid or nucleocapsid (Fig. 6 to 8). Most of the nucleocapsids which were released in this fashion possessed electron-dense nucleoids. In addition, a fine osmiophilic cloud surrounded both single and aggregated nucleocapsids (Fig. 2 to 4). A similar phenomenon was observed, albeit much less frequently, at the more convex regions of the nuclear envelope. In these instances, however, the released particles did not possess an obvious nucleoid and consisted only of capsids (Fig. 4).

Membrane-bound particles were also present in cytoplasmic protrusions of the perinuclear cisternae as well as within the lumen of RER (Fig. 5, 6). In most instances the particles situated in these parts of the hepatocyte did not possess a nucleoid (Fig. 5, 6) and only rarely were complete virions observed within the cisternae of RER. Occasionally, the envelopes of particles within such cisternae were seen fusing with membranes of the RER, releasing their content into the cytoplasm (Fig. 6).

Paranuclear collections of the amorphous electron-dense matrix were often seen at this stage, especially in bg/bg mice. Nucleocapsids with electron-dense cores were embedded in the amorphous electron-dense matrix, especially at its periphery. These aggregates were invariably situated near elements of the Golgi complex and often near the concave face of the organelle (Fig. 7 to 9). They were surrounded by vesicles and semilunar lamellae which fragmented them.
into distinct small zones (Fig. 7 to 9). As a result, nucleocapsids with electron-dense cores and a rim of electron-dense matrix were now enveloped by the outer membrane of the vesicles or lamellae which surrounded this complex cytoplasmic inclusion.

After these stages, again mitochondria were enlarged and the inner chamber of a few was now swollen. Glycogen rosettes and lipid globules were few while the concentration of lysosomal dense bodies was reduced. Bile canaliculi were dilated and occasionally electron-dense matrix material with embedded nucleocapsids was seen bordering the canalicular membrane (Fig. 8). Enveloped virus particles were seen within secondary lysosomal vacuoles by the fourth day of infection (Fig. 10). The number of virus particles within these varied from one to four but in all virions, electron-dense material surrounded the nucleocapsid(s). The size of the virus particles varied from 140 nm to 350 nm and their release at the cell surface was observed (Fig. 11) where the lysosomal vacuoles and cell membranes fused resulting in the presence of free virus both in the space of Disse as well as in bile canaliculi (Fig. 11). Small radial spikes were seen on the surface of some of these virions (Fig. 11).

Areas of focal cytoplasmic degradation were now common and some of these contained a few membrane-bound virus particles. In addition, crystalline bodies with a distinct lattice pattern were occasionally seen in the cytoplasm of infected hepatocytes.

In a few virus-containing hepatocytes, large discontinuities of the nuclear envelope were found with direct communication between nuclear and cytoplasmic contents (Fig. 12). In some of these hepatocytes heterochromatinic masses were detected in the region of the nuclear periphery but no cytoplasmic evidence of cell injury was present. In others, electron-dense matrix with embedded nucleocapsids was seen at the border between the non-enveloped nuclear region and the adjoining cytoplasm. In such instances few nucleocapsids were free in the nucleoplasm and the majority were embedded in the electron-dense matrix (Fig. 12). Moreover, most of the embedded nucleocapsids on such occasions did not possess electron-dense cores.

**DISCUSSION**

Generally, the ultrastructural features of murine hepatocytic damage induced by MCMV are similar to those described by Ruebner et al. (1964), in their original electron microscopic studies of murine hepatitis after cytomegalovirus infection. However, the stages of MCMV replication and virion production in murine hepatocytes as documented in our investigations have elucidated a mechanism which seems to be unique to this virus.

As described by others, the first structural evidence of cytomegalovirus replication is seen in the nucleus (Ruebner et al., 1964, 1965; Middlekamp et al., 1967; Fong et al., 1979, 1980; Fong, 1982). Fibrillo-granular electron-dense material, amorphous electron-dense matrices, the uncommon distinct fibrillar structures and obvious nucleocapsids all heralded replication of MCMV in infected murine hepatocytes. Fong (1982) in her studies of guinea-pig embryo cells infected with guinea-pig cytomegalovirus (GPCMV) observed that viral DNA synthesis was associated with the electron-dense fibrillo-granular material as well as the more usual fibrillar structures. It is unclear what role the amorphous electron-dense matrix plays in MCMV replication, but it seems that the more mature nucleocapsids with their electron-dense cores are often associated with it.

There appear to be two ways in which the structurally recognizable viral products can enter the cytoplasm. The more common is the budding of capsids and nucleocapsids together with the

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**Fig. 8.** Hepatocyte 4 days after infection with MCMV. Some electron-dense matrix is near the Golgi complex (arrow) while some is in the vicinity of a bile canaliculus (curved arrow). Bar marker represents 400 nm.

**Fig. 9.** As in Fig. 8. Two nucleocapsids have been surrounded by a Golgi cisterna (arrow) which has invaded the electron-dense matrix. Bar marker represents 200 nm.

**Fig. 10.** As in Fig. 8. Virus particles are seen within lysosomes (arrows). Note the osmiophilic material which surrounds the nucleocapsid. Bar marker represents 400 nm.

**Fig. 11.** As in Fig. 8. Virions, some obviously multicapsid, are being released on the cell surface (arrows). Bar marker represents 400 μm. Inset shows that the surface of some virions has a series of small spikes (arrow). Bar marker represents 100 nm.
segments of the inner membrane of the nuclear envelope into the perinuclear cisternae while the second occurs when the continuity of the nuclear envelope is lost, permitting confluence of nuclear and cytoplasmic contents. Both, however, are probably only a stage in the formation of complete virions, but only the first has been previously reported (Ruebner et al., 1964, 1965; Middlekamp et al., 1967; Fong et al., 1979, 1980; Fong, 1982).

The nucleocapsids budding into the perinuclear cisternae often possess electron-dense cores indicating that either the ‘mature’ nucleocapsids are being selected or that maturation occurs during the budding process. Capsid-like structures also seem to bud into the perinuclear cisternae but these do not contain an obvious nucleoid. Occasionally, the particles budding into the perinuclear cisternae did not seem to contain recognizable viral structures. The significance of these observations is uncertain but it seems that factors other than viral nucleocapsids influence the budding process and may also be responsible for irregularity of the nuclear periphery.

The electron micrographs demonstrate that the membrane-bound particles in the perinuclear cisternae can release their contents into intranuclear cytoplasmic projections, or less frequently the immediate paranuclear cytoplasm, the membrane of the particle fusing with the outer nuclear envelope. A few particles pass into the cisternae of the RER but their contents too can be released to the cytoplasmic ground substance by a process similar to that occurring at the outer membrane of the nuclear envelope. We have found no evidence to support the hypothesis that virus particles pass directly through the nuclear pores into the cytoplasm (Fong et al., 1979).

It seems then that mature nucleocapsids, seemingly empty capsids and possibly other virus products (as represented by buds containing only nucleoplasm) are transported across the nuclear envelope and less frequently the endoplasmic reticulum and accumulate in the

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Fig. 12. As in Fig. 8. The nuclear envelope is deficient and continuity between nuclear and cytoplasmic contents exists (arrows). Electron-dense matrix (ED) with embedded nucleocapsids is seen both in the nucleus (NU) as well as in the junction between the nuclear and cytoplasmic contents. Bar marker represents 500 nm.
Ultrastructure of cytomegalovirus hepatitis

1987

perinuclear cytoplasm. The released 'mature' nucleocapsids (those with an electron-dense core) may then embed into accumulations of the amorphous matrix which could also have formed by a similar transportation process. Alternatively, the amorphous matrix may reflect the activity of the many ribosomes which are found in the intranuclear cytoplasmic projections in which the various viral products collect. Such a process may account for the formation of the cytoplasmic inclusions which are a common feature of cytomegalovirus infection.

The interaction of the 'mature' nucleocapsid–amorphous electron-dense matrix aggregate and the elements of the Golgi apparatus appear to be the next stage of virion formation. The nature of the interaction may have some resemblance to the process of autophagocytosis. The end result is that the vesicles and the inner lamellae of the Golgi apparatus invade the nucleocapsid–amorphous matrix aggregate. The 'mature' nucleocapsids together with a rim of matrix bud into the Golgi components and become enclosed within secondary lysosomes. During this interaction multicapsid virions, which are a feature of MCMVs (Hudson et al., 1976) are produced. The virions which are now covered with membrane from the Golgi apparatus are transported within lysosomes across the hepatocytic cytoplasm and then released by emiocytosis into the extracellular space.

Possibly an important step in virus release is the entry of intracellular virions into the secretory vesicles of host cells. In this way virions can reach the extracellular space by emiocytosis. The secretory vesicles and vacuoles of the acini and ducts of salivary glands and the lysosomes of hepatocytes may serve as the ideal vehicles for such a process.

The Golgi complex appears to be involved only in infected cells in which nucleocapsids and the associated electron-dense amorphous matrix are free in the cytoplasm. Such an association was observed in murine tissues infected by MCMV (Ruebner et al., 1964; Lussier et al., 1974) but also in guinea-pig embryo cells infected in vitro with GPCMV (Fong et al., 1979) as well as infection with human cytomegalovirus (HCMV) (McGavran & Smith, 1965; Ruebner et al., 1965, 1966). Ruebner and his colleagues (1966) in their ultrastructural studies of MCMV murine hepatitis reported acid phosphatase activity around individual virus particles and in the matrix of cytoplasmic inclusions. This adds more support to the involvement of lysosomes during the course of MCMV replication.

It may be argued that the in vivo system used in these studies is likely to result in repeated infectious cycles and that the naked virions seen in the cytoplasm of infected hepatocytes represent not newly formed MCMV but uncoated extracellular virus and hence that our observations reflect virus infection and not replication. However, one would expect that in a cell in which MCMV is replicating the most frequently observed phenomena would be those of replication. But naked cytoplasmic virions are much more frequent than extracellular virus particles and the cytoplasmic inclusions are seen only in cells in which MCMV is replicating in the nucleus. Moreover, if enveloping of intranuclear nucleocapsids by both the outer and inner membranes of the nuclear envelope is a common event of MCMV maturation and intracytoplasmic transportation, why were such profiles not found although carefully searched for?

The cytoplasmic inclusions consisting of nucleocapsids embedded in an amorphous electron-dense matrix were somewhat more prominent in bg/bg mice. This may perhaps reflect the malfunction of the lysosomal apparatus of these mice (Prieur et al., 1972; Kimball et al., 1975; Vassalli et al., 1978), which may be affecting its interaction with the cytoplasmic inclusion and decreasing the piecemeal removal of virions from the main body of the inclusion.

Discontinuity of the nuclear envelope also permits the direct entry of masses of dense amorphous matrix and embedded nucleocapsids into the cytoplasm. In this instance, however, the embedded nucleocapsids do not all possess electron-dense cores, indicating a possible lack of maturation. Perhaps failure of transnuclear cisternal transport of nucleocapsids delays the maturation process. The focal disappearance of the nuclear envelope may indicate that the infected hepatocyte is in the process of division and evidence exists which shows that cytomegalovirus infection favours the entry of cells into the synthetic phase of the cell cycle (De Marchi & Kaplan, 1977; Gupta & Rapp, 1978; Muller et al., 1978; Furukawa, 1979).

Lastly, it was noticed that although MCMV replication occurred in hepatocytes, the sinusoidal cells including the Kupffer cells did not support productive viral growth. Again the
reasons for these differences are unclear especially since the mononuclear phagocytes of the murine splenic red pulp are infected soon after infection (Papadimitriou et al., 1982). This may indicate that differences exist between the mononuclear phagocytes of the spleen and those of the liver (Kupffer cells) with regard to MCMV infection.

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REFERENCES


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