Murine Cytomegalovirus Particle Types in Relation to Sources of Virus and Pathogenicity

(Accepted 27 July 1981)

SUMMARY

Murine cytomegalovirus (MCMV) preparations from mouse embryo fibroblasts and from infected salivary glands were purified on potassium tartrate density gradients and examined by electron microscopy. The cell culture virus contained multicapsid enveloped virions which are ruptured easily and account for the excess number of free capsids in these preparations. Salivary gland virus consisted of single-capsid enveloped virus and equal numbers of free capsids. Particle types were imperfectly separated on density gradients, but successful separation was achieved by filtration through 220 and 450 nm Millipore membrane filters. Naked capsids were not infectious and could not be rendered infectious by centrifugal adsorption, showing that centrifugal enhancement of MCMV infectivity is not mediated through this mechanism. Although present in excess number in (avirulent) cell culture virus preparations, naked capsids did not interfere with the action of (virulent) salivary gland virus in newborn mice.

Although murine cytomegalovirus (MCMV) is classified as a member of the herpesvirus group (Roizman et al., 1973), unlike other herpesviruses (e.g. herpes simplex types 1 and 2) the infectivity of a given virus preparation can be enhanced markedly by low-speed centrifugation during virus adsorption (Osborn & Walker, 1968; Hudson et al., 1976b). The mechanism of enhancement is unexplained. Another unusual feature of MCMV is that the lethality for suckling mice of the virulent (salivary gland passaged) strain of virus is much reduced after even one passage in cell culture and the virulence is restored by one re-passage in vivo (Osborn & Walker, 1971). Also, Hudson et al. (1976a) reported that MCMV grown in cell culture consisted of many enveloped particles which contained more than one capsid, whereas multicapsid virions were not seen in salivary gland virus. The evidence from Ficoll gradient sedimentation indicated that multicapsid virions were infectious and could be centrifugally enhanced. However, in both preparations, there were also variable numbers of naked capsids of uncertain origin and infectivity. Lussier et al. (1974) examined cell culture-grown MCMV by negative staining and reported that the majority of particles were unenveloped. Enveloped particles were rarely seen and multicapsid virions were not mentioned.

We have made further studies of the different morphological forms of MCMV and attempted to relate them to virulence and the centrifugal enhancement of infectivity. Primary mouse embryo fibroblasts (MEF) were prepared by the trypsinization of foetuses from 17-day pregnant CD1 mice. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% foetal bovine serum (FBS), 0.11% sodium bicarbonate, 100 U of penicillin and 100 μg of streptomycin. Secondary cell cultures were used.

The Osborn strain of MCMV (Chong et al., 1981) was used as a salivary gland virus (SGV) suspension, after sonication. Osborn virus was also used after 10 to 15 passages in MEF at a low multiplicity of infection (approx. 0.01 p.f.u./cell). The virus growth medium used was MEM containing 2% FBS. Cultures were frozen and thawed once 6 days after
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Table 1. *Sedimentation of CCV on linear potassium tartrate gradient*

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Predominant particle type</th>
<th>Infectivity (p.f.u./ml)</th>
<th>Relative particle concn.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top (1)</td>
<td>Single capsid enveloped</td>
<td>$1.1 \times 10^5$</td>
<td>$&lt;0.1$</td>
</tr>
<tr>
<td>(2)</td>
<td>Smaller multicapsids</td>
<td>$3.4 \times 10^4$</td>
<td>$&lt;0.1$</td>
</tr>
<tr>
<td>(3)</td>
<td>Larger multicapsids</td>
<td>$8.6 \times 10^4$</td>
<td>1</td>
</tr>
<tr>
<td>(4)</td>
<td>Naked capsids</td>
<td>$7.6 \times 10^4$</td>
<td>10</td>
</tr>
<tr>
<td>Bottom (5)</td>
<td>Denser multicapsids</td>
<td>$8.0 \times 10^4$</td>
<td>$&lt;0.1$</td>
</tr>
</tbody>
</table>

* Concentration relative to that of the large multicapsid fraction (=1.0). Similar results were obtained in two other experiments.

Infection and centrifuged at low speed to give cell culture virus (CCV). Samples were assayed by a plaque-forming technique as previously described (Chong *et al.*, 1981). On electron microscopic observation, routinely prepared CCV consisted of enveloped particles containing 1 to 15 capsids and ten times as many free ('naked') capsids. SGV stocks consisted of single capsid enveloped particles and approx. the same number of naked capsids.

For further purification, virus suspensions were centrifuged at 5000 g for 15 min and the supernatants collected. CCV particles were first pelleted at 90,000 g for 1 h in a Beckman SW27 rotor and the pellet resuspended in 2 ml virus growth medium. This was layered over a stepped potassium tartrate gradient prepared in PBSA consisting of 1 ml 35% potassium tartrate and 7 ml 10% potassium tartrate, and centrifuged at 90,000 g for 1 h. Suspension of SGV was directly centrifuged twice through a similar stepped potassium tartrate gradient. The 10%/35% interphase containing most of the virus was diluted in a small volume of PBSA and layered over a linear 15 to 35% potassium tartrate gradient in PBSA and centrifuged at 100,000 g for 3 h. Fractions of 1 ml were collected and each fraction was assayed for infectivity and the type of particles determined by electron microscopy. Fractions with peak virus infectivity were pooled, pelleted and further centrifuged in fresh gradient for 18 h. In some experiments, the linear potassium tartrate density gradients were prepared in PBSA containing 30% glycerol so that a negative viscosity gradient was formed (Chong *et al.*, 1981). All centrifugations were carried out at 4 °C and gradient centrifugations were performed in a Beckman SW36 rotor. Virus samples were examined after negative staining with 2% (w/v) potassium phosphotungstate at pH 7 in a Hitachi HU12A microscope. The number of each type of particle were counted from 10 grid squares showing satisfactory staining.

It was suspected that multicapsid virions may not be stable during the homogenization procedures involved in the harvesting of SGV and that this might account for the absence of multicapsid virions in SGV preparations. Therefore, CCV was homogenized under the same conditions as for SGV. The infectivity decreased by 50% and larger multicapsids (9 to 15 capsids) were replaced by smaller ones containing 3 to 7 capsids and there were now larger numbers of naked capsids. Routine ultrasonication, however, did not affect the morphology of the various forms of MCMV and reduction in infectivity was insignificant. These observations suggest that multicapsids are indeed not found in infected salivary glands, and that the greater numbers of naked capsids seen in CCV preparations is as a result of the disruption of multicapsid virions and release of capsids.

Herpesviruses are unstable and most purification procedures are known to destroy the infectivity of the virus (O'Callaghan & Randall, 1976). The infectivity of CCV and SGV was reduced by 50% when either was suspended in 15, 25 or 35% potassium tartrate in phosphate-buffered saline at 4 °C for 4 to 24 h. After gradient centrifugation, about 35% of the infectivity of the starting material was consistently recoverable from the fractions and virus retained its morphological integrity as examined by electron microscopy. The loss in
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Table 2. Filtration of MCMV through Millipore filters

<table>
<thead>
<tr>
<th>Filter pore size (nm)</th>
<th>Virus</th>
<th>Infectivity (p.f.u./ml)</th>
<th>Infectivity reduction (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-filtered</td>
<td>Filtered</td>
</tr>
<tr>
<td>220</td>
<td>SGV</td>
<td>7.8 x 10^5</td>
<td>6.8 x 10^4</td>
</tr>
<tr>
<td></td>
<td>CCV</td>
<td>1.6 x 10^5</td>
<td>1.0 x 10^4</td>
</tr>
<tr>
<td>450</td>
<td>SGV</td>
<td>5.2 x 10^6</td>
<td>2.8 x 10^6</td>
</tr>
<tr>
<td></td>
<td>CCV</td>
<td>2.2 x 10^6</td>
<td>2.8 x 10^3</td>
</tr>
</tbody>
</table>

* Similar results were obtained in four other experiments. Membrane filters (Millipore) were washed before use with 1:5000 versene solution, followed by calcium and magnesium-free PBS. The filtrates from test materials were assayed immediately.

Infectivity during gradient centrifugation therefore is the same for CCV and SGV, and represents mainly inactivation by potassium tartrate.

After purification of SGV on a linear density gradient only one relatively sharp peak of infectivity was detected which corresponded to the fraction containing predominantly single-capsid enveloped particles. The naked capsids were heavier than the enveloped particles and were seen as a more clearly visible light-scattering band. Although the naked capsid fraction contained 5 to 10 times more particles than the enveloped fraction, the infectivity was lower by greater than two log_{10}. This suggests that the naked capsids of SGV are non-infectious and the low level of infectivity in the naked capsid fraction was due to the enveloped particles occasionally seen.

After centrifugation on a linear density gradient, CCV also gave a single peak of infectivity but with a broader base. The infectivity and distribution of the various types of particles were as shown in Table 1. The fraction with peak infectivity consisted of mostly multicapsid enveloped particles. Less dense fractions contained smaller multicapsids with small numbers of naked capsids. These fractions were lower in infectivity which most probably reflects the number of multicapsids of various sizes in the fractions. The least dense fraction did not form a clean band and contained single-capsid enveloped particles as well as small multicapsids. The naked capsid fractions formed a clearly visible light-scattering band containing large numbers of particles, but infectivity was low. Some multicapsids appeared to sediment much faster than the naked capsids and were responsible for the low infectivity seen in denser fractions which contained very few naked capsids.

It was concluded that this method was unsatisfactory for separating multicapsids from single-capsid enveloped particles. The latter were not separated from smaller multicapsids, and the multicapsids, containing from 2 to 15 capsids, were spread over a large distance on the gradient. Naked capsids formed a distinct band, but this was very close to and contaminated the peak multicapsid band, and the separation was not improved by the use of negative viscosity gradient. It is possible that the multicapsid virions are inherently unstable, tending to rupture in situ at all stages in processing, and this would account for their presence in the less dense fractions.

Further attempts to separate particle types were made by passing CCV and SGV preparations through 220 nm and 450 nm Millipore filters (Table 2). After filtration of SGV through the 220 nm filter, 10% of infectivity was recovered in the filtrate. This showed that most of the infectious particles were too large to go through the filter pore tunnels. When 450 nm filters were used, about 50% of infectivity was recoverable from the filtrate. Electron microscope observations showed that nearly all the enveloped virions in SGV preparations contained single capsids, those with two capsids comprising no more than a few percent of the total. Enveloped particles with a single capsid (diam. 220 nm) would readily pass through filters with 450 nm pores, yet only about 50% of the original infectivity could be recovered.
This could have been due to retention of infectious virions on the membrane, but only 0.1% of infectivity could be recovered by washing the 450 nm membrane in PBSA after filtration. The 50% loss may indicate irreversible adsorption of particles to the millipore membrane, or it could be a result of virion damage by the turbulence of fluid passing through filter pores. In the case of CCV nearly all the infectious virus failed to pass through the 450 nm and 220 nm filters. The 450 nm filter would allow the passage of enveloped particles containing one or two capsids, but not those containing larger numbers of capsids. It is the latter type of particle that is particularly numerous in CCV preparations, and it is considered that these particles are infectious. Since both filtrates contained large numbers of naked capsids as seen by electron microscopy, it is concluded that these are non-infectious. Similar results were obtained using either freshly harvested or −80 °C stored virus preparations.

The infectivity (p.f.u.) of MCMV can be enhanced up to 50 times by light centrifugation during the adsorption period on MEF monolayers (Osborn & Walker, 1968). Experiments were carried out to see whether centrifugal enhancement was dependent on particle types. Multiwell plates containing MEF monolayers plus MCMV were centrifuged at 280 g at 20 °C for 45 min during the adsorption period. It was found that after a single freeze-thaw of CCV (contained in 2 % serum) the titre fell by up to tenfold, and a variable fall in titre was often encountered during storage at −80 °C. This is assumed to be due to disruption of enveloped (infectious) particles. Conceivably, it is these particles that are rendered infectious (enhanced) by low-speed centrifugation. Capsid-enriched CCV was obtained by filtration through 220 nm Millipore filters. The filtrate was then centrifuged at 90000 g for 1 h and the pellet obtained was resuspended in 1/100 of the original volume. The control virus used was the original unfiltered CCV stock virus. The original CCV preparation showed the expected level (13 ×) of centrifugal enhancement but the filtered preparation showed a much lower (4 ×) enhancement of infectivity. It is considered that the small enhancement seen in the capsid-enriched preparation was due to the presence of residual enveloped particles. Clearly, the naked capsid particles present in vast excess have not been rendered infectious by centrifugation. The same result was obtained in an experiment (data not shown) in which a capsid-enriched preparation was obtained by treating CCV with 20 % ether.

It was thought possible that the excess numbers of non-infectious capsids present in CCV interfered with the replication of infectious virus in vivo and, thereby, contributed to the loss of virulence in such preparations. In vitro experiments were carried out, using capsid-enriched CCV obtained by filtration as described above. Preparations containing about 10^7 naked capsids per ml (estimated from electron microscopic particle counts) were added to MEF to give 10 capsids per cell in 0.1 ml and incubated for 1 h at 37 °C. Cells were then washed and infected with known amounts of CCV and SGV. The results showed that pretreatment with capsids did not affect the plaque number or plaque size of either CCV or SGV preparations. It is concluded that excess capsids did not interfere with the adsorption and replication of enveloped particles.

Interference by CCV or capsid-enriched CCV was also tested for in vivo in newborn CD1 mice. Preparations of CCV were injected intraperitoneally at the same time and also 18 h before intraperitoneal inoculation with 10^3 p.f.u. SGV. This dose of SGV by itself produces a mortality of 70% and this was not affected by the presence of 10 times as much infectious CCV or capsid-enriched CCV. There were also no differences in the average survival time. These results suggested that neither the capsids nor the multicapsid virions interfere with the virulence of SGV.

The results in this study confirm the findings of Hudson et al. (1976a) that multicapsid enveloped particles were the predominant enveloped particles seen in cell culture-grown MCMV. It is surprising that these unique particles appear to be restricted to MCMV. We have extended their observations and shown that multicapsid virions account for greater than
95% of the infectivity seen in CCV preparations. In freshly harvested preparations, the multicapsids were usually intact with 'full' capsids and characteristic spikes on the envelope. Many completely intact multicapsid virions were also seen with no strain penetrating the envelope. However, damaged multicapsid virions were frequently seen and it appears that they are particularly labile, being disrupted on freezing and thawing, perhaps on storage at -80 °C, and during homogenization procedures.

Capsids completely free from enveloped particles are difficult to obtain by conventional techniques; density gradients gave imperfect separation, whereas chemicals such as ether and detergent may damage the capsids without affecting their morphology. The filtration method described here yielded much cleaner capsid preparations and it was possible to obtain good evidence that the naked capsids of MCMV are not infectious. Although the mechanism of centrifugal enhancement of infectivity remains unexplained, it was clear that naked capsids cannot be rendered infectious in this way.

The virulence for newborn mice of CCV and SGV preparations was investigated in relation to the types of virions present. There was no evidence that excess naked capsids interfered with virulence. It is conceivable that infectious multicapsid virions (CCV) are themselves less virulent than single-capsid enveloped virions (SGV). This would account for rapid loss of virulence on cell culture, and rapid recovery of virulence on passage in mice. Multicapsid virions might be less virulent in vivo because they are readily lysed in extracellular fluids, or adsorb poorly to cells or infect different target cells after inoculation. The non-neutralizing mouse immunoglobulin bound to the infectious particles of SGV preparations (Chong et al., 1981) may also play a part in virulence.

The work described here was supported by the Medical Research Council (Project Grant no. G979/511/SB). The expert technical assistance of Paul Wharton is gratefully acknowledged.

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(Received 3 April 1981)