Human Cytomegalovirus: Purification of Enveloped Virions and Dense Bodies

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SUMMARY

Enveloped virions and dense bodies of human cytomegalovirus have been purified by centrifugation, using combination negative viscosity : positive density gradients. Light-scattering bands of each component were obtained, and when these were examined by immune electron microscopy minimal cross contamination was observed.

There is an increasing awareness of the roles which cytomegalovirus (CMV) infections may play in human disease (Weller, 1971), with concurrent interest in the development of vaccines (Pagano, 1976). Studies have therefore been undertaken to investigate the structural and antigenic features of this virus (Craighead, Kanich & Almeida, 1972). During these studies it was found that, in addition to the enveloped virion, cytomegalovirus infections give rise to structures antigenically related to, but morphologically distinct from, the enveloped virion. These structures comprise electron dense material contained within a limiting membrane and because of their appearance in the electron microscope have been termed dense bodies.

Although there have recently been several attempts to obtain purified preparations of enveloped virions and of dense bodies in order to compare their polypeptide compositions (Sarov & Abady, 1975; Fiala et al. 1976; Stinski, 1976), none of the purification methods described has led to a clean separation of these two components.

We have therefore studied the purification of enveloped virions and dense bodies by a different method, namely by an extension of the principle of negative viscosity : positive density gradient centrifugation, introduced by Barzilai, Lazarus & Goldblum (1972) for the purification of foot-and-mouth disease virus and adapted for the purification of vesicular stomatitis virus by Obijeski et al. (1974).

MRC-5 human diploid cells (Jacobs, Jones & Baille, 1970) were grown to confluency in 75 cm² Corning tissue culture flasks with Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum. These cells were used for virus production at passage levels between 25 and 35. When confluent, the cells were infected with the AD 169 strain of human cytomegalovirus (Rowe et al. 1956) at a virus-to-cell multiplicity of 5. The virus inoculum was allowed to adsorb for 2 h at 37 °C, then the newly infected cells were maintained using Eagle's MEM supplemented with 2% foetal calf serum. Cell destruction became apparent at 4 days post-infection, and by 7 days post-infection approx. 50% of the cell monolayer had been destroyed. At this stage the media from the infected cells was harvested and, after removal of gross cellular debris by low speed centrifugation, was used as the starting material in the purification schedule.

Medium from three flasks was centrifuged at 20000 rev/min for 60 min in a Beckman Type 30 rotor to pellet enveloped virions and dense bodies. The pellets were resuspended in a total of 3 ml TN buffer (0.05 M-tris/HCl, pH 7.4, 0.1 M-NaCl). A 0.5 ml sample was retained for later processing for electron microscopy, and the remainder was layered on to a
9 ml negative viscosity: positive density gradient. The gradient was pre-formed in a Beckman SW41 centrifuge tube using a standard two-chambered gradient maker, with 5 ml of 30% (w/w) glycerol in TN buffer containing 15% (w/w) potassium tartrate in the feeder chamber and 4 ml of 35% (w/w) potassium tartrate in TN buffer in the mixing chamber. The gradient was centrifuged for 15 min at 40000 rev/min in a Beckman SW41 rotor, and under these conditions enveloped virions and dense bodies sedimented to form two distinct, well separated, light scattering bands. When the gradient was illuminated from above, the upper band containing enveloped virions showed a blue-silver opalesence, while the lower band containing dense bodies exhibited a distinct brown tinge. Each band was separately isolated, diluted to 3 ml with TN buffer and re-centrifuged under identical conditions using fresh gradients. This was repeated twice, increasing the duration of centrifugation to 18 h for the final gradients. Fractions from the final gradients (Fig. 1) representing the two light scattering bands were processed for electron microscopy, along with a sample of the concentrated starting material. A 0·5 ml sample of each specimen was diluted 10-fold with TN buffer then mixed with 0·2 ml of a human serum which had a titre of 64 in complement fixation tests using the AD169 strain of human cytomegalovirus. The antigen-antibody mixtures were left for 1 h at room temperature and then centrifuged for 60 min at 25000 rev/min in a Beckman SW41 rotor. The supernatants were discarded and the pellets resuspended in a small amount of distilled water. Negative staining was then carried out in the usual manner using 3% (w/w) phosphotungstic acid adjusted to neutrality with 1·0 N-NaOH.

Examination of the starting material revealed the presence of numerous aggregates consisting of both typical enveloped herpes virions and dense bodies. These aggregates
always contained both forms, in agreement with previous studies (Craighead et al. 1972), where it was shown by immune electron microscopy that enveloped virions and dense bodies of human cytomegalovirus shared common surface antigens. Examination of the two gradient fractions also revealed the presence of numerous aggregates. Those from the upper band consisted almost entirely of enveloped virions (Fig. 2a) although an occasional dense body could be found. The lower band contained complexes consisting almost entirely of dense bodies (Fig. 2b) and prolonged search was needed to locate an enveloped virion.

The purification of enveloped cytomegalovirus particles was first described by Huang,
Chen & Pagano (1973) and subsequent reports have described the purification of both enveloped virions and dense bodies by sucrose (Sarov & Abady, 1975; Fiala et al. 1976) or sorbitol and caesium chloride (Stinski, 1976) gradient centrifugation. Contrary to Sarov & Abady (1975), Fiala et al. (1976) achieved only partial separation of enveloped virions and dense bodies by centrifugation in sucrose gradients, while Stinski (1976) reported that he obtained no separation using sorbitol and caesium chloride gradients. In our hands, enveloped virions and dense bodies co-sedimented as a single band in sucrose gradients, unless first sonicated extensively, as described by Sarov & Abady (1975), when two bands are resolved. This extensive sonication resulted in an unacceptably high degree of degradation of the separated components, while the less severe sonication conditions used by Fiala et al. (1976) failed to disaggregate the components sufficiently to obtain a satisfactory separation. Huang et al. (1973) reported extensive aggregation of cytomegalovirus preparations following exposure to sucrose. This is confirmed by our own observations and contra-indicates the use of sucrose gradients for the purification of enveloped virions and dense bodies.

The separation of enveloped virions and dense bodies is made more difficult by the size variation shown by the dense bodies. Although the majority of the dense bodies in our starting material were larger and sedimented more rapidly than the enveloped virions, they ranged in size from one to two times the diameter of the enveloped virions. Whereas the enveloped virions sedimented in gradients as a narrow band due to their close size distribution, dense bodies formed a broad zone, the trailing part of which was poorly separated from the band of enveloped virions. The negative viscosity of the glycerol component of our gradients enhanced the higher sedimentation rate of the dense bodies relative to that of the enveloped virions, since the more rapidly sedimenting dense bodies encountered a continually decreasing viscosity gradient and increasing centrifugal force earlier than the slower sedimenting enveloped virions. This caused the dense bodies to accelerate away from the enveloped virions and hence enhanced the separation of these two components. The dense bodies banded at their isodense position in the positive density gradient formed by the potassium tartrate component of our combination gradient system, sharpening the otherwise broad zone of dense bodies into a narrow band. In the initial gradients the duration of centrifugation was insufficient for the slower sedimenting enveloped virions to reach their isodense position in the gradient. This was a deliberate action since we wished to achieve the maximum separation between the enveloped virions and dense bodies: if the duration of centrifugation was increased until the enveloped virions banded at their isodense position, the separation was impaired. Having achieved a high degree of separation of the two components in the initial gradients, both were centrifuged to their isodense positions in the final gradients. This eliminated any small dense bodies which might have co-sedimented with the enveloped virions in the initial gradients.

The exact nature of the dense bodies associated with human cytomegalovirus is not yet clear, and it is of interest to determine the antigenic and structural relationships of this component with the enveloped virion and its nucleocapsid. Now that a separation technique is available these and other related questions can be examined.

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