Concentration of Human Cytomegalovirus from Large Volumes of Tissue Culture Fluids

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SUMMARY

Three methods of pelleting, ultracentrifugation (95,000 g for 60 min), precipitation with polyethylene glycol 6000 (5% v/v), and with ammonium sulphate (38% w/v), were used to concentrate human cytomegalovirus (CMV) from tissue culture fluids. Maximum recovery of infectious virus particles was obtained with the polyethylene glycol (PEG) method. The precipitating activity of PEG 6000 and PEG 20000 was then compared at different concentrations. The best results were obtained with PEG 6000 at a final concentration of 5% (v/v). Changes in pH or salt concentration, treatment of the concentrates with Pronase and long periods of time at 4 °C significantly reduced the number of biologically active CMV particles recovered by PEG precipitation.

Cytomegalovirus (CMV), now acknowledged to be among the most common parasites of man, is an important cause of mental retardation (Elek & Stern, 1974). It is not surprising, therefore, that an increasing number of investigators in the field of CMV research have concluded that an effort should be made to develop a vaccine against this ubiquitous pathogen (Elek & Stern, 1974; Hanshaw, 1974).

Unfortunately, the preparation of immunogenic material for use as a possible vaccine is seriously hindered by the fact that, in contrast to other members of the herpes group, CMV is more host-cell specific, requires a much longer infectious cycle, and has distinctive thermal and chemical lability characteristics in the extracellular state (Kilpatrick et al. 1976). Furthermore, this virus is distinguished by the tendency for infectious particles to be strongly associated with cells in vitro, and repeated attempts to purify the virus from infected cells were not successful (Huang et al. 1973; Fiala et al. 1976).

However, relatively pure CMV has been concentrated from the extracellular fluid by polyethylene glycol (Forghani et al. 1976) or by velocity sedimentation (Huang et al. 1973; Fiala et al. 1976; Schmidt et al. 1976; Talbot & Almeida, 1977) and precipitation with ammonium sulphate has been employed for concentrating a variety of animal and human viruses, including herpesviruses (Mukojima & Gunven, 1973; Weliky et al. 1975). Thus it seemed interesting to investigate the possibility of using this latter method to obtain biologically active CMV concentrates, as well as to determine which of the three techniques is the most effective means of concentrating the virus from large volumes of culture medium.

Human WI-38 fibroblast cells, obtained from L. Hayflick, were cultivated in roller bottles (each with a surface area of about 640 cm²) with Eagle’s basal medium (EMB) containing 10% (v/v) foetal calf serum (FCS) and 50 μg aureomycin per ml. Confluent monolayers were infected with CMV strain AD-169, obtained from American Type Culture Collection, at a ratio of approx. 0.3 infectious particles per cell. The virus inoculum was allowed to adsorb for 2 h at 37 °C, and then EMB medium supplemented with 2% (v/v) FCS (200 ml) was added to the culture which was rotated (20 rev/h) at 37 °C for 7 days.

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Cells and debris were first removed from the aged culture fluids by low-speed centrifugation (4000 rev/min for 45 min; Beckman J-6 centrifuge). The clarified fluids in each harvest were then collected, pooled, uniformly mixed, and divided into equal volumes (1000 ml) in order to provide uniform virus preparations for concentration by the three different methods.

Pelleting of CMV by high-speed centrifugation was done as previously described by Fiala et al. (1976). The clarified fluids were centrifuged at 28000 rev/min for 60 min in a type 30 rotor of a Beckman model LS-50 ultracentrifuge at 4 °C. Concentration of CMV by PEG was done essentially as described by Forghani et al. (1976). PEG 6000 (6K) or PEG 20000 (20K), obtained from Union Carbide Canada Ltd, was added slowly to the supernatant fluid (pH 7.0) to a final concentration of 1, 5 or 10% (v/v). The suspension was kept at 4 °C, with or without stirring, and then the sedimentable phase was collected by low-speed centrifugation. Salt concentration and pH were adjusted before precipitation with PEG using solid NaCl, and HCl (6 n) or NaOH (5 n) solutions, respectively. The CMV concentrates were treated with different solutions of Pronase according to the procedure of Bronson et al. (1975). Pronase (B grade, Calbiochem) was prepared in TE (0.1 M-tris-HCl pH 7.0, and 0.001 M-EDTA) buffer and was self-digested at 37 °C for 90 min. Virus pellets, resuspended in this solution, were incubated at 37 °C for 30 min and then cooled in ice. Concentration with ammonium sulphate was performed as described above for PEG, except that an equal volume of a saturated ammonium sulphate solution (76.7%, v/v, pH 7.0) was added to the supernatant fluid to precipitate the virus. In all cases, the virus pellet was resuspended in 10 ml (1/100 of the initial volume) of TE buffer (Fiala et al. 1976).

Samples (0.025 ml) of 10-fold dilutions of the virus in EMB containing 2% (v/v) FCS and antibiotic were added to drained WI-38 monolayers in wells of a microtitre plate (no. 1-220-29TR, Cooke Laboratory Products). After 2 h, an additional 0.075 ml medium was added, and incubation was continued at 37 °C in a CO2-air atmosphere. The end-point of infectivity was read 14 days after infection, at which time the foci of round, enlarged cells were counted at 40× and 100× magnifications. Virus titre was expressed in mean tissue culture infective dose (TCID_{50}) per ml.

The amount of protein was assayed by the method of Lowry et al. (1951) using serum albumin as a standard.

The yield of CMV from ammonium sulphate or PEG precipitation was compared to the yield by conventional high-speed centrifugation. Data presented in Table 1 show that ammonium sulphate 100-fold virus concentrates obtained by ammonium sulphate precipitation are almost as infective as virus preparations made by ultracentrifugation. Thus precipitation with ammonium sulphate appears as an interesting alternative to the tedious high-speed centrifugation. But the PEG precipitation method (which also permits concentration of CMV particles from large volumes of culture fluids using only short periods of low-speed centrifugation) is preferred over the ammonium sulphate method since it gives virus preparations about five times more infective for a much smaller increase in the initial volume of infected medium. Ammonium sulphate and PEG are effective in concentrating proteins (Iverius & Laurent, 1967; Robinson & Duesberg, 1968) and, in agreement with this, ammonium sulphate and PEG concentrates contain 3.0 and 1.7 times more non-viral protein per ml than ultracentrifugation concentrates. Thus, increased precipitation of proteinaceous debris from medium supplements appears as the major disadvantage of the two precipitation methods. This contamination of the virus preparations can be significantly reduced, however, by purification on sucrose gradients (results not shown).

Since the PEG method appeared as the most convenient means of obtaining CMV particles, the virus was precipitated from infected tissue culture fluids with varying amounts of
Table 1. Recovery of infectivity and protein content during concentration of extracellular cytomegalovirus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Virus titre (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
<th>Infectivity recovered (%)</th>
<th>Protein content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified supernatant fluid</td>
<td>1000</td>
<td>1.80 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>100</td>
<td>1.02</td>
</tr>
<tr>
<td>Ultracentrifugation (95000 g for 60 min)</td>
<td>10</td>
<td>5.91 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>1.57</td>
</tr>
<tr>
<td>Ammonium sulphate (38 %)</td>
<td>10</td>
<td>4.58 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>4.48</td>
</tr>
<tr>
<td>Polyethylene glycol (5 %)</td>
<td>10</td>
<td>2.55 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>14</td>
<td>2.61</td>
</tr>
</tbody>
</table>

Table 2. Precipitation of CMV with polyethylene glycol as a function of molecular weight and concentration

<table>
<thead>
<tr>
<th>Mol. wt.</th>
<th>Concentration %</th>
<th>Virus titre (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
<th>Infectivity recovered (%)</th>
<th>Protein content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 6000</td>
<td>1</td>
<td>3.98 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>17.8*</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.53 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>24.7</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.92 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>22.0</td>
<td>4.40</td>
</tr>
<tr>
<td>PEG 20000</td>
<td>1</td>
<td>2.51 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>11.2</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.17 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>18.6</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.79 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8.0</td>
<td>5.47</td>
</tr>
</tbody>
</table>

* Total infectivity of clarified supernatant fluids: 2.2 x 10<sup>7</sup> TCID<sub>50</sub>.

PEG 6K and 20K. For this series of experiments, higher multiplicities of infection (m.o.i. = 0.5 TCID<sub>50</sub>/ml) were used, and precipitation with PEG was done without constant stirring. The percentage of starting material recovered in the concentrates was slightly increased under these conditions. The results in Table 2 indicate that the relative infectivity of the CMV concentrates generally correlates with the amount of PEG used (v/v) to precipitate the virus; and that PEG 6K is more efficient than PEG 20K to concentrate the virus. In agreement with previous reports on virus precipitation with varying amounts of PEG 6K (Adams, 1973; Bronson et al. 1975), we found that recovery of CMV from infected tissue culture fluids is optimal at a concentration of 5 % (v/v). This conclusion was reached after considering that PEG 6K (5 % v/v) concentrates are relatively more infective and less contaminated with proteins from medium supplements than other PEG concentrates. This concentration of PEG 6K was thus used for the remaining experiments as a control.

Leberman (1966) has reported that the precipitation of plant viruses is favoured by a low net charge on the virus particles or by the presence of shielding electrolyte. Therefore, the effect of pH and salt concentration on the precipitating properties of PEG 6K (5 %, v/v) for CMV was examined. Only 9.9 and 5.6 % of the infectious virus found in the clarified supernatant fluid was recovered in the concentrates following precipitation at pH 5.0 and 9.0, respectively; and the percentage of starting material declined from 24.7 to 6.6 % as the NaCl concentration was progressively increased from none to 1.0 M-NaCl. However, more protein from medium supplements were found in these virus concentrates than in the control preparations obtained at pH 7.0 without salt. The extreme chemical lability of CMV in the extracellular state (Kilpatrick et al. 1976) is probably responsible for the adverse effects of pH and salt on the recovery of biologically active CMV from tissue culture fluids.

On the other hand, Bronson et al. (1975) have shown that concentration of Rous sarcoma virus (RSV) from tissue culture fluids with PEG, with or without NaCl, results in
significant and highly variable losses caused by entrapment of virus particles in proteinaceous debris; and that treatment of concentrated preparations with Pronase (2 mg/ml) greatly enhances the recovery of virions. Experiments carried out with CMV indicate that this virus is much more sensitive to Pronase than RSV, since only 0.6% of the initial infectivity is recovered in the virus preparations treated with ten-times lower concentrations of enzyme. This implies that the PEG-Pronase method is not useful for concentrating CMV, unless more appropriate conditions of treatment with this enzyme are found.

Finally, CMV was precipitated with PEG 6K (5%, v/v) for 2, 4, 16, and 48 h, at which times 18.0, 31.6, 24.7, and 10.5% of active viruses were found. Thus recovery of infectious virus was maximal after 4 h of precipitation with PEG. At this time, the virus preparations were showing more than 90% of the protein content found in the 16 h and the 48 h PEG concentrates. Since precipitation of proteins and CMV particles with PEG is almost completed by 4 h, these data suggest that keeping the virus at 4°C for longer periods of time results in the inactivation of infectious virus (Stinski, 1976).

The nature of the processes involved in the precipitation of proteins and viruses with PEG, as well as the cause for the inactivation of CMV in PEG-containing solutions, still remain unknown. Nevertheless, the PEG concentration method, used in accordance with the present data, will most likely provide the large amount of intact virus particles required for further biochemical and immunological studies with CMV.

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REFERENCES

Short communications


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