Propagation and Purification of High-Titer Human Cytomegalovirus

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High-titered yields of human cytomegalovirus (CMV), strain AD 169, were produced in WI-38 cells in large roller bottles. Maximum plaque titers were observed by the 4th day after infection at which time infectivity in the medium was 200 times greater than that associated with the cells. Virus released into the medium was recovered by sedimentation in a sucrose gradient in a continuous-flow centrifuge rotor. Maximal viral infectivity was found at a sucrose concentration of 42%, equivalent to a density of 1.18 g/cm³. Deoxyribonucleic acid extracted from these preparations was about 80% viral and 20% cellular as judged by equilibrium centrifugation in cesium chloride density gradients.

Cytomegaloviruses (CMV) are important human pathogens (8, 16). Human strains, however, do not replicate to high titer in cell culture (15), a circumstance which has hampered both serological and biochemical characterization of these viruses. We have now developed techniques for the growth and partial purification of large yields of a human CMV strain. These methods are described in the present report.

MATERIALS AND METHODS

Cell culture. Twenty-first passage WI-38 cells in large roller bottles (surface area = 1,200 cm²) were purchased from Flow Laboratories, Rockville, Md. The cells were propagated in 200 ml of Eagle's medium supplemented with 6% fetal bovine serum.

Virus production and purification. An inoculum of human CMV, strain AD 169, confirmed by Martos et al. was obtained from Flow Laboratories (6). Roller bottles which contained confluent monolayers of WI-38 cells were infected at a multiplicity of 0.1 plaque-forming unit (PFU) per cell; after 2 hr of adsorption at 37°C, the medium was removed, and the cell sheet was washed and replaced with fresh medium. Cells and medium were harvested at the indicated times after infection. Cells were removed from the glass by trypsinization, washed in Earle's balanced salt solution (EBSS), and collected by centrifugation at 1,100 × g. CMV infectivity titers were determined by plaquing virus on WI-38 cells by the method of Plummer and Benyesh-Melnick (8). Medium virus infectivity titrations were performed on unclarified fluid diluted in EBSS. Infectivity titration of cell-associated virus was performed on undisrupted cells which were washed initially and then suspended in EBSS. When scaled-up for the production run, cell-associated virus was released from cells by sonic treatment and purified by a combination of nuclease treatment and differential centrifugation as described by Russell and Crawford (10). Supernatant fluid virus was recovered in the B-XVI continuous-flow centrifuge rotor (4). McCombs has reported a concentration of CMV by banding virus as the interface of a two-step sucrose gradient (5). In the present study, virus was isopycnically banded in a continuous sucrose gradient which was rapidly formed by diffusion in the B-XVI rotor. The sucrose solutions, which were introduced in two steps, consisted of 370 ml of 40% sucrose over 300 ml of 60% sucrose (weight for weight) in 0.1 M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride at pH 7.0. The same Tris-hydrochloride buffer was used to completely fill the rotor and to maintain a slow flow rate through the rotor while accelerating to speed. When a rotor speed of 28,000 rev/min was attained, the flow was switched to virus-containing medium and the rate was adjusted to 3 liters per hr. Centrifugation was continued for 1 hr after the medium passed through the rotor. After virus addition, one additional liter of Tris-hydrochloride buffer was flushed through the system. The speed was then reduced to 4,000 rev/min, and 60% sucrose was used to displace the rotor contents. The effluent was monitored spectrophotometrically at 260 nm and 27-ml fractions were collected. Sucrose concentration, density, and CMV infectivity titers were determined for individual fractions. Selected fractions were examined by electron microscopy. Virus was recovered from appropriately pooled fractions by sedimentation for 3 hr at 40,000 × g in a no. 21 rotor of the Spinco model L centrifuge.

Purification of deoxyribonucleic acid. Deoxy-
ribonucleic acid (DNA) was recovered from preparations of CMV by incubation at 37 C for 12 hr with 1.0 mg of Pronase per ml in the presence of 0.3% sodium lauryl sulfate (SLS) as described by Thomas, Berns, and Kelly (14). The incubation mixtures were then extracted with phenol, and DNA was dialyzed into SSC (0.15 M NaCl, 0.015 M sodium citrate). DNA concentrations were determined by assuming an optical density at 260 nm of 1.0 for a solution containing 50 μg of DNA per ml.

Equilibrium sedimentation of DNA. DNA was preparatively banded at 25 C in a CsCl solution (0.01 M Tris, pH 8.6; ρ = 1.720 g/cm³) in a no. 40 Spinco rotor at 33,000 rev/min for 60 hr. (3). Gradient fractions were collected through a puncture in the bottom of the centrifuge tube, and optical density at 260 nm was determined for each fraction. The density of selected fractions was measured. Buoyant densities of DNA components in CsCl were also determined in the model E ultracentrifuge (9). Clostridium perfringens DNA and Micrococcus lysodeikticus DNA were used as markers.

Electron microscopy. Virus was pelleted in an SW 27 rotor directly into Beem capsules by means of a special adapter designed by Smith and Gehle (11). The pellets were then fixed for 1 hr in 1% chromosomum, rinsed, washed in 0.5% aqueous uranyl acetate, dehydrated in alcohol, and left overnight in a 1:1 mixture of propylene oxide and Epon-Araldite (1). The material was then embedded in Epon-Araldite and sectioned on a Reichert OM U2 ultramicrotome. Sections from individual fractions were scanned for virus particle with a Siemens 1A electron microscope.

RESULTS

Production and purification of CMV. The time course of growth for CMV in WI-38 cells was followed by infectivity titrations to determine when maximum virus harvests could be obtained (Fig. 1). At the highest input multiplicity that was added (0.1 PFU per cell), the yield of cell-associated infectivity was optimal 3 days after infection (10⁴ PFU/ml), whereas the infectivity peak of virus released into the medium was reached on the 4th day after infection (10⁵ PFU/ml). On the 4th to 5th day, infectivity in the medium was 200 times greater.
Fig. 3. Morphology of CMV particles collected by equilibrium sedimentation in sucrose. (a) × 70,000; (b) × 70,000; (c) × 35,000; (d) × 160,000.
DISCUSSION

The production of high-titered yields of a human CMV strain has not been reported previously. With the system described, 200 times more infectious virus was present in medium than in cells when peak titers were achieved by the 4th day after infection. In a study by others of the time course of infectivity with a human CMV, there was a predominance of cell-associated virus and maximum titers were lower than those observed by us (15). Stationary monolayer cultures were employed, and the highest input multiplicity of infection used was 0.01. The greater virus yield in the present study can be attributed to a higher input multiplicity (0.1 PFU/cell) and the use of roller cultures. We have only been able to achieve high virus yields with roller cultures. The debris associated with virus separated from medium and partially purified by continuous-flow zonal centrifugation can be reduced by a rebanding or a sedimentation velocity step, and viral DNA extracted from these preparations (Fig. 4) may be additionally purified by a repeated equilibrium sedimentation in CsCl.

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Fig. 4. Equilibrium sedimentation in a CsCl density gradient of released plus cell-associated CMV DNA extracts. Peak fractions were pooled, and the densities shown were determined in the analytical ultracentrifuge.
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