Large-Volume Purification of Tumor Viruses by Use of Zonal Centrifuges

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Large volumes of fluids from selected cell cultures producing ribonucleic acid tumor viruses were processed by a double sucrose density gradient procedure using zonal centrifuges. The primary recovery utilized a Model K continuous-flow zonal centrifuge at 9 to 11 liters/hr. The virus zone from the Model K gradient was further purified on a second gradient in a B-29 rotor. The resulting viral concentrates at $2 \times 10^{11}$ particles per ml showed high purity by electron microscopy and gel analysis and were useful reagents in biochemical and immunological studies of the reverse transcriptase enzyme, virus structure, complement fixation, and other virus properties. Similar recovery methods were applied to other tumor virus systems.

The Model K zonal centrifuge (2) permits the application of the principles of continuous-flow density gradient centrifugation to larger volumes of tumor virus fluids than possible with other zonal centrifuges (4, 11, 24). This centrifuge has been used successfully in the preparation of highly purified influenza (9, 18) and rabies (12) virus vaccines. As part of the Special Virus-Cancer Program of the National Cancer Institute, we have been applying the Model K and other zonal centrifuges to the concentration and purification of a variety of murine, feline, and avian ribonucleic acid (RNA) tumor viruses propagated in cell culture. This report is a summary of the techniques used in our laboratory to prepare highly purified concentrates of these relatively high-yield viruses. The same techniques have been used for the recovery of other viruses, particularly the low-yield viruses found in cell cultures of human origin such as the ESP-1 type C virus (17) and the Epstein-Barr herpesvirus (7).

MATERIALS AND METHODS

Propagation of virus fluids. Cell cultures chronically infected with selected RNA tumor viruses and actively producing mature virus into the culture medium were chosen for large-volume propagation (Table 1).

Monolayer cultures were propagated in roller bottles (Belco Glass, Vineland, N.J.) or 32-oz (approx. 960 ml) flat-bottom flasks by using standard culture medium fortified with 5 to 10% heat-inactivated fetal calf serum. Fluids for virus recovery generally were harvested every 24 hr from cultures that were more than 50% confluent. As many as five or six harvests could be obtained per culture before signs of culture degeneration were apparent. For special purposes (increased virus infectivity, enzyme activity, or higher levels of native 60 to 70S RNA per physical particle), reduced harvest schedules were employed. With reduced intervals between fluid changes, the serum content of the culture media was reduced to as low as 2% serum for a 3-hr harvest interval.

Suspension cultures were propagated in unstirred vessels using enriched cell culture media (RPMI 1640, McCoy's 5A, Leibovitz Li5) with 10 to 15% heat-inactivated fetal calf serum. When cell counts reached $5 \times 10^4$ cells per ml, 30 to 50% of each culture was removed every 24 to 48 hr for virus fluid recovery with makeup to the original culture volume with fresh medium. This schedule generally permitted the cultures to maintain themselves at $5 \times 10^4$ to $15 \times 10^4$ cells per ml over extended periods.

All harvested virus fluids were quickly clarified by centrifugation at $4,080 \times g$ for 5 to 10 min at 4 C (Sorvall RC2-B centrifuge). The clarified fluids generally were stored frozen ($-70$ to $-90$ C) until sufficient volume was collected for virus recovery.

Primary continuous-flow zonal centrifugation. The initial virus concentration and purification was achieved by continuous-flow zonal centrifugation using a Model K centrifuge (Electro-Nucleonics, Inc., Fairfield, N.J.) with a K-3 titanium rotor and Noryl core (3.8-liter capacity). The assembled rotor and auxiliary lines were sterilized in situ by overnight exposure to 70% ethanol or isopropanol. The alcohol was displaced from the rotor and from lines using sterile air. The system then was filled and flushed with sterile cold water or buffer. Ribonu-
clease-free sucrose (Schwarz/Mann) in buffer [generally 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2), 0.001 M ethylenediaminetetraacetic acid] was the most frequently used gradient material. Two-step gradients consisting of 1,000 ml of 20% (w/w) sucrose and varying amounts of 55% sucrose were pumped into the rotor. For sample volumes of 20 to 30 liters, 500 to 800 ml of 55% sucrose was adequate; sample volumes of 50 to 80 liters required 1,000 to 1,400 ml of 55% sucrose. The objective of the two-step gradient was to have the 20% sucrose serve as a barrier between the low-molecular-weight or low-density substances in the sample virus fluid and the virus banding zone at 30 to 38% sucrose, with the 55% sucrose serving as a cushion between the virus zone and the rotor wall. An approximately linear gradient formed by diffusion between the 20% and 55% sucrose during the time period of each run.

After introduction of the gradient, the K-3 rotor was accelerated to the operating speed of 35,000 rev/min (83,000 × g, max), and the virus fluid was passed through the rotor at 9 to 11 liters per hr utilizing a peristaltic pump for sample feed (model TM35, Sigmamotor, Middleport, N.Y.). At the completion of the sample feed, the rotor generally was maintained at operating speed for an additional hour to allow banding of the virus that entered the gradient during the latter portion of the sample feed.

The rotor then was decelerated to rest and 100-ml fractions were collected through a continuous-flow recording spectrophotometer (ISCO, Lincoln, Nebr.). The sucrose concentration of each fraction was determined with a Bausch & Lomb Abbe 3-L refractometer. Recently, a continuous-flow recording refractometer (Electron-Machine Corp., Umatilla, Fla.) has been used successfully to monitor the sucrose concentration during fraction collection.

A K-10 rotor (8-liter capacity) was occasionally employed on a batch basis for the recovery of virus from 2 to 7 liters of fluid. The rotor was loaded at rest with the sample and two-step sucrose gradient, accelerated to 35,000 rev/min, and held for 120 to 180 min. Unloading and fraction collection were carried out in the same manner as described for the K-3 rotor.

Secondary zonal centrifugation using a B-29 rotor. A B-29 zonal rotor (1) with a capacity of 1,450 ml was used routinely for the second banding of the virus zone from the Model K gradient. The configuration of the B-29 rotor allows unloading of the gradient at either the center or edge with equal resolution, a feature that allowed more rapid handling of viral concentrates. The virus zone from the Model K gradient (generally five 100-ml fractions covering the range of 28 to 38% sucrose, density 1.12 to 1.17) was pooled and diluted with water or buffer to below 30% sucrose. The sample was preceded into the B-29 rotor by a 100-ml buffer overlay and followed into the rotor by either a 750-ml linear gradient ranging from 30 to 45% sucrose (Dialagrad, ISCO) or a two-step gradient of 350 ml of 30% sucrose and 400 ml of 45% sucrose. Any excess rotor capacity was filled with 45% sucrose. Centrifugation generally was at 25,000 rev/min for 180 min. Fractions (50 ml) were collected through a continuous-flow recording spectrophotometer.

The concentrated virus zone, generally 150 ml covering the range of 32 to 38% sucrose, density 1.14 to 1.17, was freed of sucrose by several methods: (i) dilution to below 25% sucrose, centrifugation to pellets in angle-head rotor tubes (80,000 × g for 120 min), and resuspension of the pellets in buffer by gentle homogenization, (ii) dialysis against buffer using standard cellulose bags, (iii) dialysis, and concentration, if desired, using membrane ultrafiltration (PM30 membrane, Amicon).

Virus concentrations were determined by electron microscopy using the semiquantitative negative staining methods of Monroe and Brandt (15). Protein assays were carried out by the method of Lowry et al. (13) with bovine albumin standards.

RESULTS AND DISCUSSION Cell culture fluids from the high-yield cell lines used in this study (Table 1) generally

| Virus type* | Cell line designation | Cell line derivation | Type of growth | Reference |
|-------------|-----------------------|----------------------|----------------|-----------|
| MuLV (Rauscher) | JLS-V10 | Normal BALB/c mouse bone marrow co-cultivated with infected bone marrow | Monolayer | 26 |
| MuLV (Moloney) | JLS-V11 | Normal BALB/c mouse bone marrow co-cultivated with infected lymph nodes | Monolayer | 26 |
| MSV (Moloney) | M-MSV (3T3) | Mouse embryo, infected in vitro | Monolayer | 23 |
| FeLV (Rickard) | F-422 | Thymus gland from leukemic cat | Suspension | 19 |
| FeLV (Theilen) | FL-74 | Lymphoid cells from leukemic cat | Suspension | 22 |
| FSV (Gardner) | G-FSV | Cat embryo, infected in vitro | Monolayer | 8 |
| RSV (Schmidt-Ruppin D) | | Chick embryo, infected in vitro | Monolayer | 6 |
| RAV-1 | | Chick embryo, infected in vitro | Monolayer | 21 |

*MuLV, FeLV = murine, feline leukemia viruses; MSV, FSV, RSV = murine, feline, Rous sarcoma viruses; RAV = Rous-associated virus.
contained more than $4 \times 10^8$ virus particles per ml under the growth conditions described above. There usually was less than 5% loss of virus in the supernatant extract from the Model K centrifuge at flow rates of 11 liters per hr or less. Flow rates above 12 liters per hr resulted in more significant levels of virus in the effluent fluids.

Several typical ultraviolet absorption profiles of gradients from Model K centrifuge runs with various RNA tumor virus fluids are shown in Figure 1. Sample feed volumes generally were in the range of 30 to 60 liters. The total amount of virus in these fluids ($10^{13}$ to $3 \times 10^{13}$ particles) caused significant ultraviolet absorption peaks in the 32 to 37% sucrose (density 1.14 to 1.17) zones.

A complete Model K centrifuge run, including decontamination, clean-up, and reassembly of the rotor and auxiliary lines, required approximately 16 hr and was carried out by a single operator on consecutive days or in a single day using a two-shift schedule.

Figure 2 presents the ultraviolet absorption profiles from the second density gradient centrifugation runs in the B-29 rotor on the virus zones from the Model K gradients shown in Fig. 1 (shaded areas). The second gradient centrifugation not only concentrated the virus, but also effected further purification, particularly in separating the virus from any "soluble" materials that often contaminated the virus zone in the Model K gradient. Also, experience with cultures contaminated with mycoplasma that banded at 40 to 44% sucrose (density 1.18 to 1.20) showed that this second

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**Fig. 1.** Gradient profile from continuous-flow recovery of various RNA tumor virus fluids in a K-3 rotor. Centrifugation and gradient conditions are given in the text. The shaded areas show the virus zones used for further purification in a B-29 rotor.

**Fig. 2.** Gradient profiles from second sucrose gradient centrifugation of RNA tumor viruses in a B-29 rotor. Centrifugation and gradient conditions are given in the text. The shaded areas show the virus zones taken for the final concentrates.
gradient purification can effect a significant separation of the mycoplasma from the RNA tumor virus zone.

Of interest was the frequent phenomenon of a small fraction (5 to 10%) of the total virus in the B-29 gradient remaining at the heavy end of the initial sample zone (25 to 30% sucrose, density 1.11 to 1.13) and not sedimenting with the principal virus band found at density 1.14 to 1.17. The virus at density 1.11 to 1.13 was morphologically similar to virus banding at density 1.14 to 1.17 and showed comparable biochemical (e.g., reverse transcriptase) and immunological (e.g., complement fixation) properties. This virus may represent a portion of the virus in the cell culture fluids that exhibits nonideal sedimentation properties because of physicochemical attractions between the virus and certain lipid or lipoprotein components in the sample zone.

The final virus concentrates from the B-29 rotor gradients, when freed of sucrose, generally were adjusted to contain $2 \times 10^{11}$ virus particles per ml and frozen at $-70 \, ^\circ C$ in small-volume portions. Such concentrates normally assayed total protein values of 0.3 to 0.6 mg per ml and showed significant levels of reverse transcriptase activity for isolation studies (20) and for assay purposes using both exogenous synthetic template (poly rA:oligo dT) and endogenous viral template (S. Aaronson and S. Yang, personal communications). The level of extractable 60 to 70S RNA was also significant in such viral concentrates (R. Gallo, personal communication). However, as reported by Bader and Steck (3) and Watson (25), the level of intact native 60 to 70S RNA per virus was increased as the cell culture harvest interval was reduced to the 3- to 4-hr range.

The viral concentrates at $2 \times 10^{11}$ particles per ml could be used as antigens in the complement fixation tests developed for the various RNA tumor viruses (COMUL, COCAL, COFAL; 10) at dilutions of 1:64 to 1:128 versus 4 antibody units with no detectable anticomplementary activity (H. Turner, personal communication). Many of these concentrates show little or no detectable deoxyribonucleic acid or ribosomal or other cellular RNA by sensitive analytical gel electrophoresis techniques (J. Bader, personal communication). Electron micrographs of a typical concentrate (Fig. 3) indicate the relatively high purity of the viral concentrates obtained by these procedures. Preparations of this type have been used for recent structural studies of Rauscher murine leukemia virus (14).

These methods have also been applied to other virus systems, including the Epstein-Barr (7) and other herpesviruses. For herpes-

![Fig. 3. Electron micrographs of Rauscher murine leukemia virus purified from tissue culture fluids by the methods described in the text. Left, thin section, $\times 35,000$; right, negative stain, $\times 35,000$.](image-url)
virus recovery, the sucrose gradients were extended to 60 to 62% sucrose (density 1.29 to 1.30) to allow banding of any unenveloped, nucleated virus in the fluids at density 1.25 to 1.26. Mature, enveloped herpesviruses banded at density 1.19 to 1.20. These higher densities compared to the typical RNA tumor viruses allowed somewhat higher flow rates of 12 to 15 liters per hr during Model K centrifuge continuous-flow operation.

Of course, one is not limited to sucrose gradients in these operations, and successful virus recoveries have been made in this laboratory using, for example, glycerol, Ficoll (Pharmacia, Piscataway, N. J.; reference 16), and Ficoll-heavy water (5) gradients.

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