NOTES

Recovery of Murine Leukemia Virus from Large Volumes of Freshly Harvested Culture Fluids by Using a Single Density Gradient

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Concentration and purification of murine leukemia virus for use as complement fixation antigens was accomplished by using a single density gradient in the model RK ultracentrifuge.

High concentrations of ribonucleic acid (RNA) tumor viruses have been purified from large volumes of tissue culture fluid in the model K or RK ultracentrifuge by using double density gradient techniques, or by precipitation before centrifugation (4, 12). Smith and Bernstein (11) have shown that pelleting avian tumor viruses from cell cultures consistently gave higher recovery of infectious virus than ammonium sulfate precipitation or polyethylene glycol concentration, and that virus infectivity remained relatively constant through a single zonal purification procedure but decreased after a second density gradient purification using a BXIV zonal rotor. The desirability of utilizing a single density gradient purification procedure without prior chemical concentration, therefore, seems evident.

We sought to produce large volumes of purified AKR virus that had been processed within a short time after harvest, using as little treatment or manipulation as possible to minimize denaturation. Using the RK-3 rotor in a single purification step, RNA tumor virus was recovered from large volumes of tissue culture media, which gave consistently good reactivity as antigens in complement fixation (CF) tests.

The NIH/3T3 cell line derived from NIH Swiss embryo cultures infected with a virus isolate from AKR, L-1 strain, murine leukemia virus (MuLV) (9) was used in this procedure. This RNA tumor virus causes no cytopathogenic effect in chronically infected cultures and buds from the cell cytoplasm into the surrounding growth medium. Monolayer cultures were propagated in roller bottles (Belco Glass, Inc., Vineland, N.J.) by using Eagle minimal essential medium fortified with 2 to 10% fetal bovine serum. Spent fluids were harvested from cultures more than 40% sheeted, pooled, and clarified of whole cells by centrifugation at 4,080 \( \times g \) for 8 to 10 min (Ivan Sorvall, Newtown, Conn.). Daily harvests (up to four per week) from 100 to 150 roller bottles provided the sample volume for centrifugation, which was done immediately after collection.

The model RK ultracentrifuge developed by Anderson et al. (1) utilizes interchangeable rotor cores that permit zonal separations by batch or continuous-flow operations, or clarification or pelleting without gradient. Purified fractions of poliovirus, influenza virus, the trachoma agent Chlamydia trachomatis, nuclear polyhedral insect virus, and plant cell chloroplasts have been prepared by using the RK-II and J-1 rotors in the model RK centrifuge (2, 3). The continuous-flow RK-3 titanium rotor with Noryl core (1.6-liter capacity) was utilized throughout this experiment in the model RK ultracentrifuge (Electro-Nucleonics, Inc., Fairfield, N.J.). Gradient material was ribonuclease-free sucrose (Schwarz/Mann) in 0.05 M sodium citrate, pH 6.7. Two-step discontinuous gradients were used to achieve high resolution, allowing diffusion to form approximately linear gradients during the time of the run. Five hundred milliliters of 20% sucrose and 700 ml of 50% sucrose provided good separation for sample volumes ranging from 9 to 22 liters.

At the operating speed of 35,000 rpm (90,000 \( \times g \) maximum), the sample flow rate was held to 5 liters/h or less to allow sufficient particle
retention time within the rotor to achieve a high percentage of virus recovery. Gradient fractions (50 ml) were removed and stored at 4 C overnight and were concentrated the following morning. Fractions covering the range of 30 to 38% sucrose (density, 1.13 to 1.165) were diluted to below 25% sucrose with sterile deionized water and centrifuged in angle-head rotor tubes (Beckman Instruments, Palo Alto, Calif.) at 44,000 x g for 180 min. The virus pellets were aspirated in 0.017 M sodium citrate, pH 6.7, homogenized briefly with a motor-driven Teflon-glass homogenizer, and diluted to a final concentration of not less than 2 x 10^11 virus particles/ml as determined by electron microscopic examination (7).

Virus pools were treated with 1:4,000 formalin (Baker) at 4 C for 7 days with a daily swirling of the flask. At the end of this period, the pool was dispensed in 2-ml portions and frozen in vapor-phase liquid nitrogen, except for a small volume that was retained on wet ice for immediate CF testing.

Formalin-treated virus concentrates were tested by the microtechnique of Sever (10) against the following: serum from goats immunized with electrofocused MuLV group-specific (gs) antigen (goat anti-MuLV), supplied by R. Gilden, Flow Laboratories, Rockville, Md.; serum from NIH Swiss mice hyperimmunized with twice-banded AKR virus; and pooled sera from Fischer rats carrying murine sarcoma virus-Moloney tumor transplants (MSV-30). The latter serum has been reported to be reactive against envelope and gs antigens of all murine C-type viruses in CF tests (5). CF titers for untreated virus concentrates were compared with Tween-ether- or Triton X-100-disrupted preparations.

The data in this report are derived from 25 zonal runs carried out over a period of approximately 3 months. The conditions for every run were kept as identical as possible throughout and the operations were performed by the same laboratory personnel. The AKR-infected cell cultures grown under the conditions described above varied in their daily virus yields from approximately 9 x 10^8 to 6 x 10^9 virus particles/ml, averaging about 2.5 x 10^8 virus particles/ml. Sample volumes ranged from 9 to 22 liters. Figure 1 shows a typical ultraviolet absorption peak at a density of 1.16, indicating good separation of virus from other materials present in the sample after centrifugation in the RK-3 rotor. Approximately a 1,000-fold concentration was necessary to achieve final virus concentrations of 2 x 10^11 to 2.5 x 10^11 virus particles/ml. Protein assays (6) comparing predensity gradi-

ent samples with these concentrates showed more than a 20-fold reduction in total protein in some cases. Figure 2 shows these concentrates to be relatively free of cellular debris after the single density gradient purification. Reverse transcriptase assays (8) of random samples by using the endogenous viral template gave [3H]deoxythymidine triphosphate incorporation values greater than 2 x 10^8 counts per min per ml.

The major gs protein of the RNA tumor viruses is thought to be internal to the virus membrane. Virus disruption with Tween-ether of Triton X-100 detergent caused an increase in the CF titer when assayed against gs monospecific antiserum and, therefore, gave an indication of the intactness of the virion before treatment. For our purposes, the criteria for virus satisfactory for use as a CF antigen were demonstration of at least a fourfold increase in titer after disruption, with a minimal titer of 1:512. Of 25 lots evaluated against goat anti-MuLV gs antiserum, 17 gave titers of 1:512 or greater and 23 showed a fourfold or greater titer increase. Of nine lots evaluated against rat anti-MSV-30 antiserum, six gave titers of 1:512 or greater.
Fig. 2. Electron micrographs of purified AKR virus concentrated from RK-3 centrifuge gradients. Negative stain (2% phosphotungstic acid). × 30,675.
and all showed a fourfold or greater increase in titer. However, of 25 lots evaluated against mouse anti-AKR MuLV antiserum, only 15 gave a titer of 1:64 or greater and 3 showed a twofold or greater increase in titer. More than 70% of the lots evaluated met the above requirements when tested against either goat anti-MuLV gs serum or rat MSV-30 serum, with little or no anti-complementary activity. Approximately half of these lots had titers greater than 1:512 after disruption (data not shown), and almost all showed at least a fourfold increase in titer after disruption. This "release" of reactivity was not demonstrated in tests using the NIH Swiss mouse serum made against whole AKR virus particles, presumably because only external envelope antigens were available for antibody production. CF titers of disrupted virus versus this antiserum averaged approximately eightfold lower than against the goat anti-MuLV gs or rat MSV-30 sera.

Using the above criteria, we evaluated 25 lots of purified AKR virus by the CF method and, with few exceptions, found that these preparations gave repeatedly good antigenic reactivity. In addition, preparations tested against gs-specific antiserum before and after disruption indicated that virus particles purified in the manner described were not grossly damaged by the purification procedure.

On the basis of prior work (12), we know that additional purification steps would eliminate even more cellular proteins that are present in the "virus zone" of a single density gradient. However, other studies (11) have shown that in certain instances it may be more desirable to eliminate multiple-step purification procedures. Because of the flow rates involved (approximately 5 liters/h), the capacity of the gradients, and the relative ease of centrifuge decontamination, disassembly, and set up, it is possible to prepare purified virus concentrates of freshly harvested virus conveniently on a daily basis by using the RK-3 rotor as the principal purification tool. Using this method, we have repeatedly recovered most of the virus particles present in spent culture fluids while concentrating and freeing them from substantial amounts of cellular debris at the same time.

Because the virus prepared in this study was formalin treated, no infectivity evaluation was done by us. However, on the basis of data presented by others (11), it is likely that the single density gradient purification of freshly harvested supernatant fluids, which we described, would be a useful procedure to apply in instances when large volumes of infectious RNA tumor virus are desired. Experiments will be done in our laboratory to examine this possibility.

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