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Production of "Rapid-Harvest" Moloney Murine Leukemia Virus by Continuous Cell Culture on Synthetic Capillaries

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Moloney murine leukemia virus was harvested automatically within 60 min of release from chronically infected NIH/3T3 cells (clone 1) cultured on bundles of synthetic capillaries. Production of virus as measured by a determination of reverse transcriptase activity and by the XC syncytia assay demonstrated that highly infectious "rapid-harvest" virus was recovered from NIH/3T3 cells (clone 1) grown for periods of up to 10 days.

Studies of a number of workers (2, 4, 5, 19) have shown that the genome and structural components (proteins) of RNA tumor virions change significantly during the period of virus formation. Duesberg et al. (5) reported that chick cells infected with avian sarcoma virus release particles that initially contain 35S RNA and that this RNA matures to a genomic length of between 60 and 70S. A decrease in the number of virion proteins and an increase in the endogenous activity of reverse transcriptase were described by Cheung et al. (2). The work of Rothenberg and Baltimore (17) has indicated that the in vitro synthesis of DNA transcripts by reverse transcriptase is facilitated by the use of detergent-disrupted virions that were harvested shortly after their release from the cell surface membrane (as well as the use of a high nucleoside triphosphate concentration and a low Mg^{2+} concentration). Preparations of type C virions that have been isolated within 2 h of their release from the cell surface have been termed "rapid-harvest" virus (2, 17).

It has also been demonstrated that type C virions harvested within 2 h of budding from the cell surface have as much as 10 times more infectivity than virions harvested from the same cells at 24-h intervals, as determined by the focus assay (21) and XC syncytia assay (9, 23). This change of infectivity has been correlated with alterations in the size and homogeneity of the RNA genome (1, 8, 11).

The production of large quantities of rapid-harvest virus by using conventional roller bottles or culture flasks is extremely tedious, costly, and subject to a substantial contamination problem due to the frequency of manual refeeding. Equipment has been described (12, 22) for the automatic harvesting of cultures growing in roller bottles. Such equipment is expensive and uses large quantities of medium.

An alternative procedure for the recovery of rapid-harvest virus was suggested by the work of Chick et al. (3), who demonstrated that large quantities of insulin were produced when pancreatic β cells were grown on bundles of synthetic capillaries. Synthetic capillaries have also been used for the continuous assay of respiratory products from mitochondrial suspensions (16). Therefore, we sought to apply this methodology to the production of rapid-harvest RNA tumor virus and report here the first use of synthetic capillaries for the continuous large-scale recovery of Moloney murine leukemia virus (M-MuLV).

A system that facilitates the automatic rapid harvest of RNA tumor virus is shown schematically in Fig. 1. The synthetic capillary unit (Fig. 1, A) consisted of a sealed plastic cylinder (10 by 1 cm) containing approximately 150 hollow polysulfone capillary fibers (Amicon 3S100). The spongelike body of each fiber was composed of numerous reticulations and cavities opening progressively outward (20), providing a total surface area of approximately 60 cm^2/capillary unit to which cells readily ahered. The NIH/3T3 cell line (clone 1) described by Fan and Paskind (7) was used in this work. This cell line is chronically infected with M-MuLV and continually releases virus particles ino the cell supernatant. Clone 1 cells were inoculated into the space around the capillary fibers by suspending a trypsinized monolayer of 10^7 cells in 2 ml of culture medium and injecting the cells through one rubber septum in the top port of the plastic capillary cylinder (Fig. 1, A) while, at the same time, medium was being withdrawn through the other rubber septum. Cells were then passed gently from one syringe to the other to distribute the cells evenly over the entire outer surface area of the capillary fibers. Cells growing throughout the 10-day period of viral production never impeded the perfusion of medium from the capillary lumen to the outer capillary surfaces. The use of a laminar

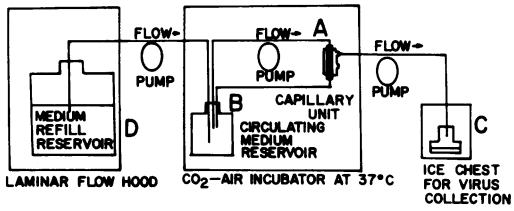

FIG. 1. *System for producing rapid-harvest M-MuLV. Synthetic capillaries (A) were inoculated under a sterile laminar flow hood with NIH/3T3 cells (clone 1) chronically infected with M-MuLV. Each capillary unit was then immediately transferred to a 10% CO_2-air incubator at 37°C along with the circulating medium reservoir (B) containing 750 ml of Dulbecco modified Eagle medium (Gibco) with 10% fetal calf serum (GIBCO) and 10 μg of Gentamicin (Schering Corp.) per ml. Having allowed 4 h for cell attachment to the outer surface of the capillaries, the medium was then continuously pumped clockwise (2 ml/min) from the circulating medium reservoir through the inner lumen of the capillaries and back to the reservoir. The synthetic capillaries (Amicon 3S100) had a nominal cutoff for molecules of a molecular weight greater than 100,000, and medium was able to freely perfuse from the lumen to outer capillary surfaces where it fed the attached cells. M-MuLV spontaneously released into the perfused medium by the clone 1 cells was immediately withdrawn (4 ml/h) from the capillary unit and collected on ice (C). Virus was sampled at 24-h intervals, and medium was replaced every 2 days with fresh medium from the medium refill reservoir (D). Silicone tubing was used throughout to provide oxygenation for the closed circulating system. A second synthetic capillary unit was run in parallel; however, for clarity, it is not shown in this diagram.*

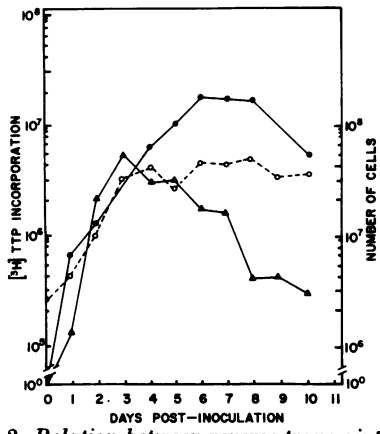

FIG. 2. *Relation between reverse transcriptase activity and growth of clone 1 cells. Assay conditions were modified from Fan and Baltimore (6) and Rapp and Nowinski (15). Portions (70-ml) of culture medium from clone 1 cells were cleared of cell debris by centrifugation at 10,000 × g for 10 min at 4°C. M-MuLV was then harvested from the culture medium by centrifugation at 82,500 × g in a Beckman 45Ti rotor for 1 h at 4°C and suspended in 350 μl of 0.01 M Tris-hydrochloride (pH 7.6). Virus contained in 15 μl of this solution was disrupted with an equal volume of 0.2% Nonidet P-40 for 10 min at 4°C, and to this suspension was added 70 μl of the reaction mixture at 37°C. The total reaction mixture was comprised of 50 mM Tris-hydrochloride (pH 8.3), 45 mM KCl, 0.2 mM $MnCl_2$, 30 mM dithiothreitol, 0.5 unit of absorbancy at 260 nm of poly(rA)·poly(dT)$_{12-18}$ annealed at a molar ratio of 1:1, and 20 μM [^{3}H]TTP (specific activity, 3.7×10^{10} cpm/μmol; New England Nuclear Corp.). The reaction was terminated by removing 10-μl samples from the reaction mixture to 0.5 ml of 0.01 M EDTA (pH 8.0) at 4°C after 0 and 12 min. Stripped yeast RNA (50 μl) was immediately added (200 μg/ml) as carrier. The incorporation of [^{3}H]TTP into trichloroacetic acid-precipitable material was determined by adding 0.5 ml of 25% trichloroacetic acid to the terminated reaction samples at 4°C. After 30 min, the samples were spotted on glass-fiber filters (Whatman GF/C), washed five times with 20 ml of 2% trichloroacetic acid, and dried for 30 min, and the contained radioactivity was determined in Redisolv IV (Beckman Instruments), using a Packard Tri-Carb scintillation spectrometer. The mean reverse transcriptase activity was determined as counts per minute of [^{3}H]TTP incorporated in 12 min for the total quantity of virus produced from three synthetic capillary units (●) or from three T-75 flasks (▲) in each 24-h period. Variation from the mean never exceeded 10%. The number of cells in T-75 flasks (○) was determined with a hemacytometer, using flasks grown under conditions identical to those used to obtain reverse transcriptase activity. All counts were performed on duplicate T-75 flasks, with variation never exceeding 10%.*

flow hood for culture inoculation and medium replacement (Fig. 1, B and D) eliminated contamination, which has been a serious problem with other synthetic capillary systems (16, 20). For these reasons, the system shown in Fig. 1 was well suited for the continued propagation of clone 1 cells.

Rapid harvest of the resulting virus was accomplished by the perfusion of medium from the lumen to the outer capillary surfaces, where it bathed the cells and carried recently released M-MuLV into the extracapillary space. The medium containing M-MuLV was continually pumped into a reservoir maintained at 0°C (Fig. 1, C). Because the volume of the extracapillary space was 2 ml and the pumping rate from the capillary unit was 4 ml/h, the majority of medium containing virus was replaced within 30 min.

The efficacy of this procedure for the production of rapid-harvest M-MuLV was measured by using the exogenous reverse transcriptase assay (6, 15, 24). As shown in Fig. 2, reverse transcriptase activity (as measured by the incorporation of [^{3}H]TTP) increased immediately after the inoculation of clone 1 cells and reached a maximum on day 6 before eventually decreasing. The decreased titer observed after day 8 was possibly due to a reduction in the mitotic activity of the

clone 1 cells as the surface of the synthetic capillary became completely filled with cells, thus reducing their rate of proliferation. This could not be confirmed directly since the actual number of clone 1 cells at any particular time could not be determined, as this cell line could not be removed from capillary units with trypsin. However, the work of Panem and Schauf (13) and Paskind et al. (14) has demonstrated that the release of type C virions is dependent upon the mitosis of chronically infected cells. In addition, we have shown that the reverse transcriptase activity of chronically infected clone 1 cells was dependent upon their growth in T-75 culture flasks (Fig. 2).

Hence, the improved growth of clone 1 cells in capillary units (compared with their growth in T-75 flasks) was probably responsible for the total reverse transcriptase activity observed in 10 days (7.9×10^7 cpm of [^{3}H]TTP), exceeding by a factor of 5 the total reverse transcriptase activity observed in 10 days with T-75 flasks (1.6×10^7 cpm of [^{3}H]TTP). This occurred even though the total surface area in the 3S100 capillary unit (60 cm^2) is less than the 75-cm^2 area of T-75 flasks. It has been suggested (3, 10, 20) that the three-dimensional growth of cells on capillary fibers facilitates extensive cross-feeding, which is not as extensive in cultures growing on the flat surfaces of roller bottles and culture flasks, and that cross-feeding allows continued cellular proliferation to higher cell densities (3, 10, 20).

Infectivity of virus isolated from synthetic capillary units was determined by using the XC syncytia test (18) with rapid-harvest M-MuLV collected on ice during the previous 24-h period. As shown in Fig. 3, the viral titer increased progressively from day 1 after inoculation with chronically infected clone 1 cells and by day 8 had reached a maximum of greater than 10^9 PFU/capillary unit. These data demonstrated that M-MuLV produced by synthetic capillary units was infectious and exhibited the high infectious titer (greater than 10^7 PFU/ml) characteristic of rapid-harvest virus (9, 21, 23). The data from this in vivo assay closely paralleled the results obtained with the reverse transcriptase assay (Fig. 2), confirming that the optimal period for harvesting M-MuLV from the synthetic capillary units was from days 4 to 11 inclusive. In addition, all of the M-MuLV harvested from capillary units and T-75 flasks during this period was subsequently purified by sucrose density gradient centrifugation and shown to possess the characteristic density (1.15 to 1.16 g/cm^3) of type C RNA tumor virions (6).

These data indicate that this synthetic capillary system (Fig. 1) allowed the continuous production of rapid-harvest RNA tumor virus, which was automatically collected on ice within 30 min of synthesis in quantities of approximately 100 ml/24 h per capillary unit. To obtain an equal amount of 30-min rapid-harvest virus from a T-75 culture flask requires the collection of seven individual portions. In addition, the synthetic capillary system also allows an immediate and precise change in the rate at which virus can be harvested simply by adjusting the pump setting governing the rate of collection of virus from the capillaries. This suggests an efficient means for studying the maturation of the RNA tumor virus genome at varying times after formation.

The use of synthetic capillary units for the production of rapid-harvest RNA tumor virus has been demonstrated to be an alternative to roller bottle and culture flasks. We are presently investigating the large-scale harvesting of M-

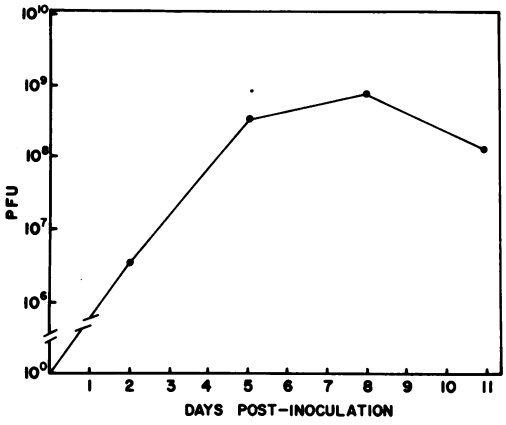

FIG. 3. *XC syncytia test using rapid-harvest M-MuLV produced by synthetic capillary units. Test conditions were modified from Rowe et al. (18) and Rapp and Nowinski (15). Petri dishes (60-mm) were seeded with 10^5 NIH/3T3 cells and incubated in 5 ml of medium at 37°C in a 10% CO_2-air incubator. After 24 h, the cells were pretreated for 1 h with Polybrene (20 μg/ml of medium) and infected with 0.4 ml of M-MuLV serially diluted in Polybrene (20 μg/ml of medium). During the next 2 h, the dishes were rocked slowly at 20-min intervals to evenly distribute the virus. Fresh medium without polybrene was then added (5 ml), and the cells were incubated for 4 days until they became confluent. At this time the NIH/3T3 cells were irradiated with a lethal amount of UV light, using a germicidal lamp (20 s at 20 cm), and immediately overlaid with 2×10^6 viable XC cells. The medium was replaced after 24 h, and 72 h later the cells were fixed in absolute methanol and stained with Giemsa to score for syncytia. Uninfected controls were always negative. The data show the mean of the total number of PFU for three synthetic capillary units in each 24-h period. Variation from the mean never exceeded 15%.*

MuLV from a synthetic capillary unit (Amicon H1P100), which has a lumen surface area 20 times larger than the 3S100 units used here.

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