Research Report

Production of Minimally Disturbed Synchronous Cultures of Hematopoietic Cells

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INTRODUCTION

Research on cell growth control and the cell cycle often requires a reliable technique for examining large quantities of cells at precisely defined stages during the mitotic cycle. One experimental approach has involved efforts to obtain synchronized cell populations, using either induction or selection procedures to generate cells at equivalent stages (3,5,11,14,15,17). Induction techniques attempt to convert an entire exponentially growing culture into a population in which growth and division are synchronized. An array of induction procedures have been reported, such as starvation and resupplementation of a required culture medium component; periodic changes in temperature, nutrition or light; and transient addition of inhibitors of specific macromolecular syntheses. Techniques of this type can be used to draw correlations with respect to the cycle stage that responds to the treatment, but they do not yield information on the mitotic cycle of cells in steady-state growth since growth is disturbed and the totality of cell processes may not be synchronized (1,3,4,11,15,18).

To overcome the problems associated with induction techniques, methods have been developed for the selection of cells at specific stages in the division cycle from exponentially growing cultures. The major selection procedures for the production of synchronous mammalian cell cultures entail collecting cells of either fixed age by detachment of mitotic cells from a culture growing on a surface (16) or fixed size by centrifugal elutriation (3,5,10, 14,19). Both

methods have proven to be successful for generating synchronously growing cultures, although there have been a few reports of potential disturbances, as judged by elongation of the first cycle after treatment (5,14,19). Many years ago, our laboratory developed a selection technique for bacteria designed for examining the undisturbed, steady-state bacterial cell cycle (6,8). In this technique, which eventually became known colloquially as the "baby-machine", cells are cultured on a surface such that when a cell divides, one daughter cell remains adhered to the surface while the other daughter is released. As a result, newborn cells are shed continuously from the surface for use in cell cycle and synchronous growth research applications (2,7,9,12,13).

This paper describes the application of the baby-machine process to mammalian hematopoietic cells. The technique is extremely simple to perform and produces minimally disturbed synchronous cells of very high quality.

MATERIALS AND METHODS

Mouse L1210 lymphocytic leukemia cells (ATCC CCL219) and human leukemic MOLT-4 cells (ATCC CRL 1582) were cultured in spinner flasks in CO₂-independent L-15 medium supplemented with 2 g/L dextrose and 10% FBS (both from Invitrogen, Carlsbad, CA, USA). Human U937 cells (ATCC CRL 1593) were cultured in 75-cm² culture flasks in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. The cell adhesives, compounds used to adhere the cells to

ABSTRACT

A method is described for producing sizable quantities of synchronously dividing, minimally disturbed mammalian cells. Cultures were grown immobilized on surfaces such that cell division within the population resulted in the continuous release of synchronous newborn cells. As judged by the quality and duration of synchronous growth, cell size distributions, and DNA compositions, newborn mouse L1210 cells grew with a very high level of synchrony without overt evidence of growth disturbances. The technology should be applicable to a variety of hematopoietic cells, as evidenced by similar results with human MOLT-4 and U937 cell lines.

Figure 1. Apparatus for production of newborn cells. Step 1: cross-sectional view of the membrane filter holder described in the text. Step 2: configuration of the holder during operation of the apparatus. The inset shows an illustration of immobilized cells growing and dividing on the inverted membrane surface.

Figure 2. Theoretical and experimental concentration of newborn cells in the effluent from a membrane-bound population of L1210 cells. (A) Idealized mitotic cycle age distribution is shown for an exponential-phase culture containing no dispersion in generation times of individual cells. (B) The solid line reflects the theoretical concentration of cells in the effluent from a membrane-bound culture with the age distribution shown in panel A and assuming the generation time is 10 h and one of the two newborn cells formed at division is always shed from the surface. The experimental curve (filled circles) shows the number of cells in consecutive samples of the effluent from a membrane-bound culture of L1210 cells growing in L-15 medium at 37°C. The membrane was coated with 20 $\mu\text{g/mL}$ poly-D-lysine, and a 234-mL culture containing 3×10^5 cells/mL was filtered onto the surface. Samples (4 min) were collected from the effluent at the indicated times.

the membrane filters, were either poly-D-lysine (30–70 kDa; Sigma-Aldrich, St. Louis, MO, USA) or concanavalin A (Sigma-Aldrich) at 20 $\mu\text{g/mL}$ in PBS (Invitrogen). Each experiment was initiated with 100–350 mL culture containing 2 to 3×10^5 cells/mL growing exponentially. Cell concentrations and size distributions were determined with a model ZB Coulter electronic particle counter, containing an aperture tube with a 100 μm diameter orifice, and a Channelizer® model 256 (Beckman Coulter, Hialeah, FL, USA). Cellular DNA distributions were determined with a FACScan™ flow cytometer (BD Biosciences, San Jose, CA, USA) after staining ethanol-fixed cells for 60 min in a solution of 50 $\mu\text{g/mL}$ propidium iodide and 100 $\mu\text{g/mL}$ RNAase A.

RESULTS AND DISCUSSION

The Technique

The basic apparatus consisted of a funnel that can accommodate a 142-mm diameter membrane filter (Figure 1, step 1). Although any funnel arrangement can be used, the current experiments employed a 150-mm diameter funnel machined from polymethylmethacrylate, similar to that used previously for *E. coli* (6). A 142-mm diameter, 0.22- μm pore size nitrocellulose membrane filter (GSWP142; Millipore, Bedford, MA, USA) was supported by a stainless-steel screen (Millipore) and clamped with plastic gasket rings between the lower funnel and an upper polymethylmethacrylate ring (13 cm, i.d.; 15 cm, o.d.). Just before use, a solution of cell adhesive, chosen for each experiment, in 50 mL PBS was poured onto the filter and drawn through by vacuum at a rate of about 1 mL/s. The membrane filter was then washed by drawing 100 mL PBS rapidly through the filter. A culture of cells (100–350 mL) growing exponentially was poured on top of the filter, and the medium was drawn through by vacuum at about 1 mL/s. Filtration was stopped when a small amount of the culture remained above the membrane, to avoid drying the cells, and the remaining fluid (about 20 mL) was poured off. The filter holder was invert-

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ed and placed on top of a second plastic funnel resting on a ring stand (Figure 1, step 2). The top of the holder was then filled with approximately 200 mL fresh culture medium (although preconditioned medium has also been used successfully) and connected to a peristaltic pump. The filter holder was aligned so that the drops from the effluent passed freely through the hole in the lower funnel. This arrangement maintained the temperature on the filter. The medium was pumped from a 1-L bottle containing a filtered vent at a rate of 15 mL/min for the first 2 min to remove loosely attached cells and 2 mL/min thereafter. The entire experiment was performed in a 37°C incubator. The pump and culture media were held on a

rack above the apparatus in the incubator. All of the fresh medium was equilibrated with a 5% CO₂ atmosphere, as were the samples collected from the effluent for synchronous growth. In the case of experiments using RPMI 1640, the entire filter apparatus was maintained in a 5% CO₂ atmosphere.

Any style filter holder can be used as long as the elution medium flows uniformly over the underside of the filter when the holder is inverted to bathe the surface-bound cells fully and to ensure that the released newborn cells are flushed from the surface. A funnel that held about 200 mL culture medium above the filter after it was inverted worked well because it introduced a slight bow in the filter, resulting in uniform medium flow and formation of a single drop from the center of the filter. This was best achieved with 0.22- μ m pore size membrane filters and a medium flow rate of 2 mL/min. Larger pore-size filters and/or slower pump rates did not flush the surface as well and should not be used.

Properties of the Newborn Cells

The concentration of cells in the effluent from a baby-machine is a function of the age distribution of the cells in the mitotic cycle in the exponentially growing population initially attached to the membrane filter surface (Figure 2A). The theoretical idealized concentration of newborn cells in the effluent from a baby-machine is given by the mitotic cycle age distribution in reverse (Figure 2B). The first immobilized cells to divide and release one daughter (time 0) would be those that were closest to division at the time of attachment to the membrane. As younger and younger cells reach the division stage and release a daughter, the concentration of cells in the effluent increases, reflecting the increasing frequency of their parents in the original culture. At the end of the first generation of growth on the membrane, twice as many newborn cells are released because there were twice as many newborn cells as cells about to divide in the original ex-

Figure 3. Cell size distributions of exponential-phase and baby-machine-generated L1210 cells. (A) Size distributions, shown as Coulter Channelizer channel numbers, in a sample from an exponentially growing culture containing 3×10^5 cells/mL (interrupted line) and in a sample collected after 3.5 h elution in the experiment shown in Figure 2B (solid line). (B and C) Size distributions in samples collected after 10 and 20 h elution.

Figure 4. Synchronous growth of newborn L1210 cells. (A) The effluent from the experiment described in Figure 2 was collected between 3 and 3.5 h and placed in a 75-cm² culture flask. Samples were taken from this synchronous culture at the indicated times, beginning at the end of collection of the effluent (time 0) for determination of cell concentrations. DNA distributions were determined by flow cytometry at the cell cycle ages indicated by the arrows during synchronous growth. The cells of different ages were obtained by collecting consecutive 10-min samples from the effluent starting at time 3.5 h and incubating each sample for different times in the cell cycle. The inset shows a comparison of the DNA distribution in an exponential-phase culture to that in a sample of the effluent. (B) Cell size distributions during synchronous growth of L1210 cell samples taken at the indicated times from the synchronously growing culture shown in panel A.

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ponential-phase population. At this time, all of the original bound cells have divided once and now begin to divide for the second time, again starting with the oldest cells. Thus, the theoretical idealized cell concentration in the effluent consists of a saw-toothed curve varying 2-fold in each generation.

The experimental result with L1210 cells mimicked the theoretical curve except for the overall increase in the curve with time (Figure 2B). This increase is a reflection of the frequency with which both daughter cells remain attached to the surface after division. The gradual rather than abrupt variations in the curve are consequences of the dispersion in generation times of individual cells (6). The generation time of cells on the membrane was about 10 h, as given by the time intervals between either the peaks or the midpoints of the decreases in the elution curve. In this experiment, approximately 5×10^7 total cells were loaded on the membrane, occupying about 50% of the 100-cm² surface, which appeared optimal, thereby yielding approximately 5×10^7 newborn cells in each generation.

Evidence that the cells released from the immobilized population were virtually all newborn is based on size distributions, synchronous growth curves, and cellular DNA distributions. The size distribution of the cells in a sample of the effluent collected at 3.5 h was narrow with a mean size corresponding to the very smallest and youngest cells in the exponential-phase culture before attachment (Figure 3A). The size distributions remained essentially unchanged for at least 20 h (Figure 3, B and C), except for the small number (about 5%) of larger cells. As shown, most of these larger cells were double the size of the newborn and appear microscopically as two newborn cells adhered together. The explanation for the doublets is unknown but likely resulted from two released cells touching and adhering during the division of adjacent immobilized cells or during passage along the membrane surface. In any event, the presence of cells other than newborn in the effluent is rare for at least 20 h.

The high quality and duration of synchronous growth of the newborn

cells were evident in measurements of cell concentrations versus time (Figure 4A), with the decay in synchrony being a natural consequence of the variation in interdivision times of individual cells. As expected, cells early in synchronous growth (1.5 h) contained G1 content of DNA, those about midway through the synchronous growth cycle (4.5 h) were in S, and those in the process of synchronous division were in either G2 or G1 (7.5 h) (Figure 4A). This synchronous progression through DNA replication continued in the same manner through at least the start of the third cell cycle. The inset in the figure shows a comparison between the DNA distributions in the exponential-phase culture bound to the membrane and the newborn cells eluted at 3.5 h.

The time course of changes in cell size distributions in the newborn cell cultures again demonstrated the high level of synchronous growth (Figure 4B). The small cells at time 0 gradually increased in size uniformly during syn-

Figure 5. Cell size distributions during synchronous growth of MOLT-4 and U937 cells. (A) MOLT-4 cells were grown in L-15 medium and attached to a poly-D-lysine-coated filter. Size distributions are shown at 0, 10, 20, and 30 h of synchronous growth of a sample collected between 4 and 4.3 h elution. (B) U937 cells were grown in RPMI 1640 medium and attached to a concanavalin A-coated filter. Size distributions are shown at 0, 8, 16, and 20 h synchronous growth of a sample collected between 3 and 4 h elution.

chronous growth until they reached double the newborn cell size and then began to divide at approximately 7 h. By 8 h, the cells were about midway through synchronous division (Figure 4A), as evidenced by the bimodal size distribution corresponding to a mixture of newborn cells and cells just before division (Figure 4B). By 10 h, essentially all of the cells had divided to yield a single peak at approximately newborn size. This same pattern was clearly evident through the second synchronous growth cycle as well. Even at the start of the third cycle, the synchrony of the cells was apparent.

Consistent with the premise that the methodology is applicable to a variety of hematopoietic cells, experiments with human leukemic MOLT-4 and U937 cell lines again exhibited clear synchrony (Figure 5). The small newborn cells at time 0 gradually increased in size during synchronous growth until they reached double the newborn cell size and underwent synchronous division, shown at 20 h for MOLT-4 and 16 h for U937. Our experience indicates that the procedure should work well with a variety of hematopoietic cells, both established lines and activated normal cells, employing an adhesive for which the particular cells have an affinity (such as charged polymers, attachment factors, or antibodies).

Finally, large numbers of synchronous cells can be obtained with this procedure by collecting consecutive samples of the effluent for about one-tenth of the generation time and growing the newborn cells to the desired stage in the mitotic cycle. Furthermore, the possibility of any disturbance of the cells that could arise from either the change from suspension growth to surface-bound growth or elution with fresh medium can be obviated by growth on the surface for several hours before collecting such samples. However, adverse growth disturbances have not been detected thus far, as judged by the quality, duration, and multi-cycle repetition of synchronous growth and division.

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